University of Alberta

Cell adhesion-related signaling molecules in embryo development

by

Marc Steven Ridyard

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of **Doctor of Philosophy**.

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For mum, dad, Paul and Leon.
You're always there to provide support when things get tough.
I couldn't have done this without you.

Abstract

The work presented here is focused on the localisation, expression and possible functions of three proteins, focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2) and paxillin, during the early stages of chick embryo development. FAK and Pyk2 are cytoplasmic protein tyrosine kinases implicated in signal transduction from integrin adhesion receptors. Paxillin is a multi-domain cytoskeletal protein that binds to both FAK and Pyk2 and may act as an adaptor molecule to anchor these and other proteins in a signaling complex. Results show that during gastrulation in the chick embryo each of these molecules is spatially regulated and shows differing distribution patterns from each other. Embryo cells grown in culture show localisation of FAK in cell-to-substratum adhesive sites and to the nucleus, also FAK expression and activation is influenced by changes in the composition of the substratum. In the embryo, FAK shows no localisation to specific adhesion sites or to the nucleus. Reducing the level of FAK expression in culture by antisense oligonucleotide treatment does not affect levels of apoptosis but affects cell spreading and shape. Comparison of FAK, Pyk2 and Paxillin protein expression *in vivo* and *in vitro* shows each molecule is independently regulated, with tissue specific changes occurring when embryo cells are grown in culture. In summary, these signaling molecules are specifically regulated and are therefore likely involved in early embryogenesis, however, the localisation and expression of these molecules is different *in vivo* and *in vitro* suggesting the cell architecture and organisation of signaling molecules at adhesive sites *in vitro* may not be a reflection of their organisation *in vivo*. FAK appears to be involved in regulation of cell shape and possibly migration rather than apoptosis in chick embryo cells in culture, possibly reflecting its role in the developing embryo.

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Table of Contents

INTRODUCTION 1

INTRODUCTION 2

LITERATURE REVIEW 4

FOCAL ADHESION KINASE 5

FAK protein structure 5

FAK homologs and splice variants 8

FAK+ 8

FAK-related non-kinase 8

Activation of FAK by integrins 10

Activation of FAK by non-integrin signaling 12

Receptor tyrosine kinases (RTK's) 13

G protein-coupled receptors 14

Oncogenic transformation 16

Other activators of FAK 17

FAK-related proteins 17

Proline-rich tyrosine kinase 2 17

FAKB 19

CELL ADHESION AND INTRACELLULAR SIGNALING 19

Integrins and intracellular signaling 19

Inside-out signaling 20

Outside-in signaling 20

The cytoskeleton and focal adhesions 21

Signaling through FAK 26

Regulation of cell motility 26

The MAP kinase pathway 27

Apoptosis 29

Signaling through Pyk2 31

CELL ADHESION AND EMBRYOGENESIS 32
Early vertebrate embryogenesis 32
Gastrulation in the chick embryo 32
Integrins and regulation of morphogenesis 35
FAK expression in embryonic tissues 36
FAK localisation in embryonic tissues 38
SUMMARY 40
AIMS OF THE THESIS 41
MATERIALS AND METHODS 42
ANTIBODIES 43
EMBRYO DISSECTION AND HOMOGENISATION 43
CELL CULTURE 43
CELL CULTURE IMMUNOCYTOCHEMISTRY 44
INTERFERENCE REFLECTION MICROSCOPY 45
WHOLE EMBRYO IMMUNOCYTOCHEMISTRY 45
WHOLE EMBRYO ULTRASTRUCTURAL IMMUNOCYTOCHEMISTRY 46
PROTEIN CONCENTRATION ASSAY 47
ELECTROPHORESIS 47
IMMUNOBLOTTING 47
IMMUNOBLOT STRIPPING 48
IMMUNOPRECIPITATION 49
CO-IMMUNOPRECIPITATION 49
TUNEL 50
ANTISENSE OLIGONUCLEOTIDES 51
STATISTICAL ANALYSIS OF DATA 51
EXPRESSION AND LOCALISATION OF ADHESION-RELATED SIGNALING MOLECULES 53
EXPRESSION OF FAK IN THE TISSUES OF THE GASTRULATING CHICK EMBRYO 54
FAK localisation in cultured cells 54
FAK LOCALISATION IN SECTIONED EMBRYOS 56

Ultrastructural localisation of FAK 59
Characterisation of antibodies on immunoblots 61
Immunoblot analysis of FAK in embryonic tissues 64
Immunoblot analysis of FAK in the primitive streak 66
Immunoprecipitation of FAK 68
Comparison of FAK expression in vivo and in vitro 70
EXPRESSION OF PYK2 IN THE TISSUES OF THE GASTRULATING CHICK EMBRYO 72
Pyk2 localisation in cultured cells 72
Pyk2 localisation in sectioned embryos 74
Comparison of Pyk2 expression in vivo and in vitro 76
EXPRESSION OF PAXILLIN IN THE TISSUES OF THE GASTRULATING CHICK EMBRYO 78
Paxillin localisation in cultured cells 78
Paxillin localisation in sectioned embryos 80
Comparison of paxillin expression in vivo and in vitro 83
SIGNALING THROUGH FAK *IN VITRO* 85
EFFECT OF SUBSTRATUM COMPOSITION *IN VITRO* ON FAK EXPRESSION AND PHOSPHORYLATION 86
Fibronectin substratum 86
Laminin substratum 88
Matrigel substratum 90
IMMUNOPRECIPITATION OF FAK UNDER NATIVE CONDITIONS 92
EFFECTS OF REDUCED FAK PROTEIN EXPRESSION AFTER ANTISENSE OLIGONUCLEOTIDE TREATMENT 94
EFFECT OF OLIGONUCLEOTIDE TREATMENT ON PROTEIN EXPRESSION AND LEVELS OF APOPTOSIS. 95
EFFECTS OF SERUM LEVEL ON FAK AND PYK2 EXPRESSION AND APOPTOSIS 99
EFFECT OF OLIGONUCLEOTIDE TREATMENT AND SERUM STARVATION ON LEVELS APOPTOSIS 102

EFFECT OF OLIGONUCLEOTIDE TREATMENT ON FAK, PAXILLIN AND ACTIN LOCALISATION ____ 104

DISCUSSION ____ **109**

DISCUSSION ____ 110

FUTURE DIRECTIONS ____ 123

REFERENCES ____ **125**

List of Tables

Table 1. Effect of oligonucleotide treatment on cell area and polarisation.....108

List of Figures

Figure 1. A diagram showing the structural domains of FAK and the related proteins Pyk2 and FRNK.....6

Figure 2. A model of a focal adhesion.....23

Figure 3. A diagrammatic representation of a gastrulating chicken embryo.....33

Figure 4. Cultured chick embryo cells immunolabeled for FAK.....55

Figure 5. Sections through a stage five chick embryo immunolabeled for FAK.....57

Figure 6. Sections through the primitive streak immunolabeled for FAK.....58

Figure 7. Ultrastructural localisation of FAK in chick embryo cells.....60

Figure 8. Immunoblot comparison of different anti-FAK antibodies.....62

Figure 9. Relationship between protein level and band density on immunoblots.....63

Figure 10. FAK expression and phosphorylation in the tissues from the stage 5 chick embryo.....65

Figure 11. FAK expression in the tissues from the stage 5 chick embryo including the primitive streak 67

Figure 12. Immunoprecipitation of FAK from whole embryo lysate.....69

Figure 13. Comparison of FAK expression levels in the tissues of the stage 5 chick embryo and in cultures grown on fibronectin.....71

Figure 14. Cultured chick embryo cells immunolabeled for Pyk2 and DNA.....73

Figure 15. Sections through a stage five chick embryo immunolabeled for Pyk2.....75

Figure 16. Comparison of Pyk2 expression levels in the tissues of the stage 5 chick embryo and in cultures grown on fibronectin.....77

Figure 17. Cultured chick embryo cells immunolabeled for paxillin.....79

Figure 18. Sections through a stage five chick embryo immunolabeled for paxillin.....81

Figure 19. Sections through the primitive streak immunolabeled for paxillin.....82

Figure 20. Comparison of paxillin expression levels in the tissues of the stage 5 chick embryo and in cultures grown on fibronectin.....84

Figure 21. FAK expression and phosphorylation in germ layer cultures grown on fibronectin.87

Figure 22. FAK expression and phosphorylation in germ layer cultures grown on laminin.... 89

Figure 23. FAK expression and phosphorylation in germ layer cultures grown on Matrigel...91

Figure 24. Immunoprecipitation of FAK and associated proteins.....93

Figure 25. Localisation of oligonucleotides after treatment of cultured cells.....96

Figure 26. Effect of oligonucleotides on protein expression.....97

Figure 27. Effect of oligonucleotides on levels of apoptosis in culture.....98

Figure 28. Detection of apoptotic cells in cultures supplemented with 0% or 10% serum....100

Figure 29. Analysis of FAK and Pyk2 expression in cells cultured with 0% or 10% serum...101

Figure 30. Effect of oligonucleotides and serum starvation on levels of apoptosis.....103

Figure 31. Effect of oligonucleotides on the localisation of FAK and paxillin.....106

Figure 32. Effect of oligonucleotides on the actin cytoskeleton.....107

List of Abbreviations

ATP,	adenosine triphosphate
BSA,	bovine serum albumin
Ca^{2+},	calcium
CADTK,	calcium-dependent tyrosine kinase
CAKβ,	cell adhesion kinase beta
CAM,	cell adhesion molecule
cAMP,	cyclic adenosine monophosphate
Cas,	Crk-associated substrate
cDNA,	complementary deoxyribonucleic acid
CE,	chicken embryo
CIPB,	co-immunoprecipitation buffer
CMF,	calcium magnesium free
Csk,	C-terminal Src kinase
DNA,	deoxyribonucleic acid
DTT,	dithiothreitol
dUTP,	deoxyuridine triphosphate
ECL,	enhanced chemiluminescense
ECM,	extracellular matrix
EDTA,	ethylenediamine-tetraacetic acid
EGF,	epidermal growth factor
EGTA,	ethyleneglycol-bis (b-amino ethyl ether) N,N'-tetra-acetic acid
FAK,	focal adhesion kinase
FAT,	focal adhesion targeting
FBS,	fetal bovine serum
FGF,	fibroblast growth factor
FITC,	fluorescein isothiocyanate
FRNK,	focal adhesion kinase-related non-kinase
GDP,	guanosine diphosphate

Graf,	GTPase for Rho associated with FAK
Grb2,	growth factor receptor binding protein 2
GTP,	guanosine triphosphate
IGF,	insulin-like growth factor
IPB,	immunoprecipitation buffer
IRM,	interference reflection microscopy
kDa,	kilodaltons
LPA,	lysophosphatidic acid
MAPK,	mitogen-activated protein kinase
mRNA,	messenger RNA
PAGE,	polyacrylamide-gel-electrophoresis
PBS,	phosphate buffered saline
PDGF,	platelet derived growth factor
PI,	phosphatidylinositol
PKA,	protein kinase A
PKC,	protein kinase C
PLC,	phospholipase C
PTK,	protein tyrosine kinase
Pyk2,	proline-rich tyrosine kinase-2
RAFTK,	related adhesion focal tyrosine kinase
RNA,	ribonucleic acid
RTK,	receptor tyrosine kinase
SDS,	sodium-dodecyl-sulphate
SH2,	Src-homology 2
SH3,	Src-homology 3
Shc,	Src homology collagen
Sos,	son of sevenless
SSC,	saline sodium citrate;
TBS,	tris-buffered saline
TdT,	terminal deoxynucleotide transferase

TGF,	transforming growth factor,
TNFα,	tumour necrosis factor alpha
TTBS,	TBS with Tween
TUNEL,	terminal deoxynucleotide transferase mediated dUTP-biotin nick-end labeling,
Tween,	polyaxyethylenesorbitan monolaurate
VEGF,	vascular endothelial growth factor

Chapter 1

INTRODUCTION

INTRODUCTION

During the development of a unicellular egg into a multicellular embryo, the cells undergo many processes, such as growth, migration, differentiation and apoptosis, in a coordinated manner to realise the final body plan of the adult. Many of the signals that coordinate the spatial and temporal events of morphogenesis come from extracellular sources such as growth factors, hormones and cytokines. The cells detect many of these signals through numerous different receptors on the cell surface. The binding of a receptor to its extracellular ligand initiates a stimulus within the cell that influences cell behavior. In the case of growth factors the receptors belong to the family of receptor tyrosine kinases. These receptors have intrinsic kinase activity, allowing them to propagate a series of reactions within the cell that can ultimately affect gene expression with a resulting effect on morphogenesis (Seedorf 1995).

Although cells adhere to the extracellular matrix (ECM) through receptors in the plasma membrane, these receptors were not previously thought to activate signaling cascades within cells. The ECM is made up of many different glycosaminoglycans, proteoglycans and glycoproteins that form a framework on which cells can adhere and it was originally thought that adhesion to the ECM provided only mechanical support for cells and a suitable surface upon which cells can migrate.

The main family of receptor molecules for the ECM is the integrins. Integrins are transmembrane heterodimers that bind to ECM molecules outside the cell and to the microfilament cytoskeletal network inside the cell (Dedhar and Hannigan 1996). There are approximately 22 different integrin heterodimers known, each of which has specificity for one or more of a variety of ECM molecules. *In vitro*, integrins localise to specific sites of adhesion on the ventral surface of the cells called focal adhesions. The ends of actin microfilaments associate with integrins in focal adhesions and the main intracellular function of integrin receptors was thought to be a mechanical one through their association with the microfilament network, providing a means of anchoring the cytoskeleton to allow control of cell shape.

Recent work has revealed that integrin receptors not only act as mediators for physically interacting with the ECM but also transduce signals into the cell in a similar

manner to other surface receptors. It was discovered that integrins associate with cytoskeletal proteins, but there are also a number of enzymes associated with integrins *in vitro*. Since integrins themselves do not have intrinsic enzyme activity, these enzymes provide a means through which integrins can activate signaling cascades and affect cell behavior in an analogous manner to growth factor receptors.

The first enzyme found to be activated by integrins was focal adhesion kinase (FAK; Schaller et al., 1992). FAK is a cytosolic protein tyrosine kinase that is activated by integrin engagement with the ECM and interacts with several known cytosolic molecules involved in signal transduction. The exact role of FAK in cell function is still not clear although its localisation to sites of cell adhesion has suggested a role in regulating formation or turnover of those adhesion sites, thereby influencing cell adhesion and motility. It has also come to light that FAK is a possible point of cross talk between integrin and non-integrin-activated signaling pathways (Rozengurt 1995). Numerous growth factors can activate FAK leading to the possibility that these growth factors could modify the cells adhesive behavior and also, that integrin adhesion could modify growth factor-associated signaling pathways.

Other integrin regulated enzymes now known include: integrin-linked kinase (Hannigan et al., 1996) and proline-rich tyrosine kinase 2 (Lev et al., 1995). With different isoforms of these proteins also expressed there is a growing array of signaling molecules activated in response to adhesion that may be involved in regulation of cell behavior.

In the context of embryonic morphogenesis, these signaling molecules provide a means by which cells can respond to changes in their surrounding ECM, through changes in adhesion via integrins, allowing the ECM to influence the pattern of morphogenesis within the embryo. The work presented here is focused on examination of the expression and possible function of the signaling molecules FAK, Pyk2 and paxillin. A greater knowledge of the regulation of these molecules and the functions that they have in embryonic cells will allow a greater understanding of how the changes in adhesion to the ECM can influence embryogenesis.

Chapter 2

LITERATURE REVIEW

FOCAL ADHESION KINASE[1]

Focal adhesion kinase (pp125^{FAK} or FAK) is a protein-tyrosine kinase (PTK) and was the prototype member of a new PTK family (Schaller et al., 1992). It is associated with intracellular signaling cascades initiated when the integrin family of adhesion molecules engages extracellular matrix (ECM) molecules (Schaller and Parsons 1993; Richardson and Parsons 1995). Early studies showed that plating cells onto an integrin binding substratum such as fibronectin, or cross linking integrins with antibodies lead to the activation of FAK (Hanks et al., 1992; Schaller et al., 1992). In cultured cells, integrins localise to specific sites of strong adhesion termed focal adhesions (Izzard and Lochner 1980). FAK derives its name from the fact that initial immunocytochemical studies showed that FAK also localises to focal adhesions in cultured cells (Hanks et al., 1992; Schaller et al., 1992). These initial studies indicated that integrins were not purely adhesion molecules with a structural role in organising the cytoskeleton but that they could also activate signaling cascades with FAK playing a central role in integrin signaling.

FAK protein structure

FAK was first identified when FAK cDNA was isolated from chicken embryo fibroblasts transformed by the Rous sarcoma virus (Schaller et al., 1992). These studies showed FAK was a structurally unique PTK because it possessed large N and C-terminal regions either side of the central catalytic domain (Figure 1). These non-catalytic regions contained no sites for membrane association and possessed no Src-homology 2 (SH2) or Src-homology 3 (SH3) domains used in protein-protein interactions. Since these initial studies several regions of these non-catalytic domains have been identified as having important functional roles.

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[1] *A version of this chapter has been published in, Ridyard, M. S. and Sanders, E. J. (1999) Potential roles for focal adhesion kinase in development. Anat. Embryol. 199:1-7*

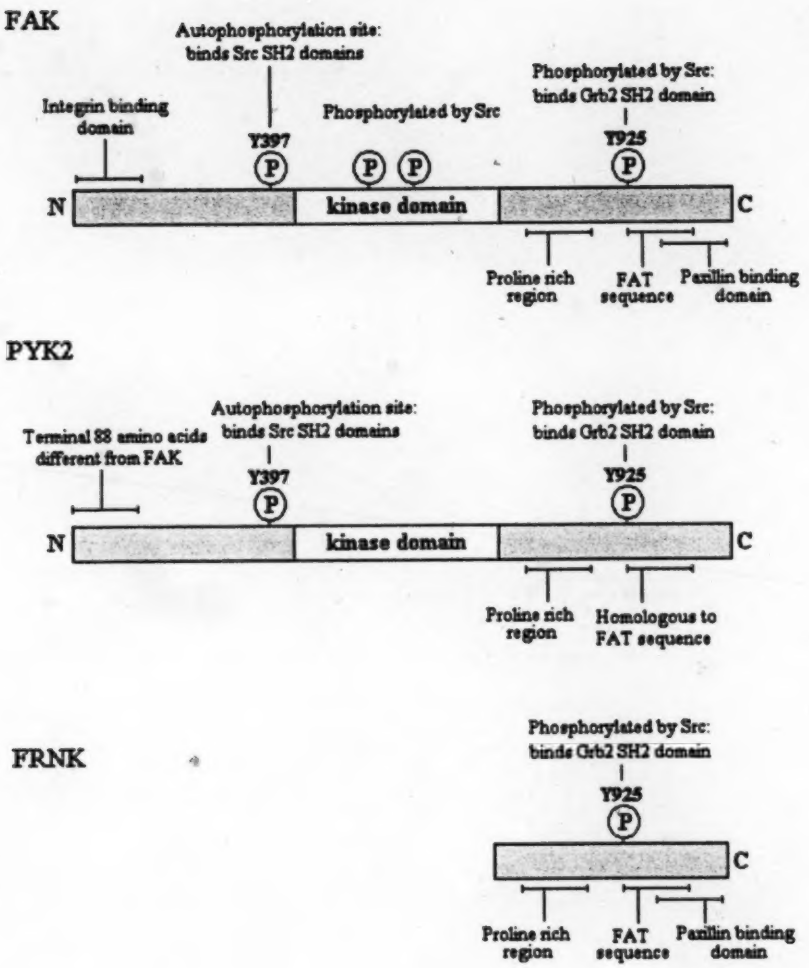

Figure 1. A diagram showing the structural domains of FAK and the related proteins Pyk2 and FRNK.

Studies on the chicken FAK have revealed several sites within the non-catalytic regions that are important functional sites. Using deletion mutations, an amino acid sequence between residues 853 and 1012, at the distal end of the C-terminal domain, was identified as a site necessary for efficient localisation of FAK to focal adhesions (Hildebrand et al., 1993). This region, known as the focal adhesion targeting (FAT) region, was shown to be required for FAK to localise to focal adhesions and was able to localise a cytosolic form of Src to focal adhesions when a chimeric Src protein containing the FAT sequence was formed. The FAT sequence is thought to be a site for protein-protein interaction, through which FAK could bind to a protein present in focal adhesions, thus directing FAK to focal adhesions.

A second sequence between residues 904 and 1054 of the C-terminal non-catalytic domain was identified as the binding site for the protein, paxillin (Hildebrand et al., 1995). Paxillin is a 68kDa cytoskeletal protein that localises to focal adhesions and so could be responsible for directing FAK into focal adhesions. The same region of FAK was identified as binding to paxillin in the human homolog of FAK (Tachibana et al., 1995). The paxillin binding region of FAK and the FAT region extensively overlap, raising the possibility that paxillin directs FAK into focal adhesions by binding to the FAT region of FAK. However, by removing the terminal 13 residues of the C-terminal non-catalytic domain of FAK, FAK loses the ability to bind to paxillin but still localises to focal adhesions. This suggests that the FAT region and the paxillin-binding site can be separated and that paxillin is not required for localisation of FAK to focal adhesions (Hildebrand et al., 1995).

Sequences in the N-terminal non-catalytic domain have also been identified as functional sites within FAK. FAK has been reported to bind, through its N-terminal domain, directly to cytoplasmic sequences of the β1, β2 and β3 integrin subunits *in vitro* (Schaller et al., 1995). This is significant due to the fact that integrin engagement can activate FAK.

The FAT sequence and paxillin-binding region in the C-terminal domain and the integrin-binding region in the N-terminal domain are all possible functional sites through which FAK could localise to focal adhesions. It is not currently known which site, or sites,

are important in the localisation of FAK or, indeed, which sites are involved in the activation of FAK.

FAK homologs and splice variants

Since the initial studies in chick embryo cells, FAK cDNA's have been isolated and characterised from mouse (Hanks et al., 1992) rat (Burgaya and Girault 1996), human (André and Becker-André 1993), canine (Tremblay et al., 1996) and *Xenopus laevis* (Hens and DeSimone 1995; Zhang et al., 1995a) tissues. These homologs exhibit between 89-97% identity to the chicken FAK at the amino acid level, showing FAK is highly conserved between species.

FAK+

The fact that FAK has been shown to be expressed in nearly all tissues examined and is highly conserved between species suggests it has a fundamental and possibly similar function in most cell types. A possible divergence in FAK function comes from studies showing the existence of different splice variants of FAK. Alternative forms of FAK have been observed in human (André and Becker-André 1993) and several different forms in rat (Burgaya and Girault 1996) where, interestingly, the alternatively spliced forms are expressed mainly in neural tissue. The original variant of FAK cloned in rat contained a three amino acid insertion in the FAT sequence and was designated FAK+. A comparison between FAK and FAK+ showed no difference in their levels of phosphorylation but the change in amino acid sequence of the FAT region of FAK+ may result in a change in its intracellular localisation. Two other insertions near to the autophosphorylation site give rise to further variants which show increased phosphorylation levels (Burgaya et al., 1997). These differences in neuronal isoforms of FAK may enable FAK to function in neural specific signaling pathways.

FAK-related non-kinase

A non-catalytic variant of FAK, termed FAK-related non-kinase (FRNK), has been identified in chicken. Isolated FRNK cDNA was found to encode for a protein of molecular weight 41-43kDa that is identical to the C-terminal non-catalytic region of FAK (Figure 1). FRNK mRNA has been identified in cells suggesting the gene encoding FAK

also produces separate mRNA transcripts encoding FRNK. Thus, FRNK appears to be expressed from the same gene, but as a separate protein from FAK, rather than being produced as a result of cleavage of existing FAK protein (Schaller et al., 1993). FRNK, like FAK, localises to focal adhesions in cultured cells, supporting the fact that sequences within the C-terminal domain of FAK are important in localising FAK to focal adhesions. FRNK also becomes phosphorylated upon cell adhesion to the ECM. The 43kDa form of FRNK represents the phosphorylated protein after ECM attachment and the 41kDa form of FRNK is the dephosphorylated form. The sites of phosphorylation on FRNK have been identified as serine residues 148 and 151 and protein kinase A (PKA) may be the serine kinase responsible for their phosphorylation (Richardson et al., 1997b).

The function of FRNK has been examined by overexpressing FRNK in chicken embryo (CE) cells and studying its effects on cell adhesion and spreading (Richardson and Parsons 1996). It was found that overexpression of FRNK resulted in a decrease in the tyrosine phosphorylation of FAK, also, focal adhesion formation was inhibited and the CE cells spread at a slower rate. Thus, FRNK appears to function as an inhibitory regulator of FAK activity. Since FRNK is identical to the C-terminal non-catalytic domain of FAK it is possible that it actively competes for the same sites as FAK within the focal adhesion, effectively blocking FAK entry into the focal adhesion. However, FRNK did not affect the ability of FAK to localise to focal adhesions. The inhibitory effect of FRNK on FAK is suggested to be a result of FRNK competing for a common binding partner that is required for FAK signaling (Richardson and Parsons 1996). As mentioned earlier, FRNK is phosphorylated on serine residues after cell adhesion to the ECM, but the level of phosphorylation of FRNK has no effect on its ability to inhibit FAK tyrosine phosphorylation (Richardson et al., 1997b). The ability of overexpressed FRNK to inhibit focal adhesion formation and cell spreading can be overcome by co-overexpression of wild-type FAK or Src, both of which lead to increased tyrosine phosphorylation of paxillin (Richardson et al., 1997a).

Many stimuli result in FAK phosphorylation and activation whereas FRNK is a possible negative regulator of FAK phosphorylation. It is not known which proteins FRNK binds to, and consequently, which proteins are involved in its inhibition of FAK

activity, but it appears to inhibit focal adhesion formation and cell spreading by inhibiting tyrosine phosphorylation of FAK, preventing its association with Src and subsequent phosphorylation of paxillin.

Activation of FAK by integrins

Interest in FAK was first raised when it was observed to become activated when cells were plated onto surfaces coated in integrin ligands or anti-integrin antibodies (Hanks et al., 1992; Schaller et al., 1992). Integrins have no intrinsic kinase activity and FAK was thought to be an integrin associated kinase, through which, integrins could activate signaling cascades.

Adhesion of cells to the ECM, through integrins, is associated with the increased tyrosine phosphorylation of FAK (Schaller et al., 1992; Nojima et al., 1995). This increase in tyrosine phosphorylation is now known to be directly related to the increased kinase activity of FAK and is a result of autophosphorylation (Guan and Shalloway 1992; Schaller et al., 1994; Calalb et al., 1995). Autophosphorylation of FAK occurs on a specific tyrosine residue, tyrosine 397, which lies close to the ATP binding site (Schaller et al., 1994). Tyrosine 397 is located in the N-terminal domain proximal to the central catalytic domain, a somewhat unusual location for an autophosphorylation site since they are commonly found in highly conserved regions within the catalytic domain in other PTK's.

The mechanism by which FAK is activated and autophosphorylation is brought about is not clear at present. It was initially thought that physical interaction with proteins in the focal adhesion, possibly integrins themselves, could result in activation of FAK. The fact that FAK binds directly to the cytoplasmic region of β integrin subunits *in vitro* supports the possibility of activation by direct interaction with integrins (Schaller et al., 1995). Phosphorylation of FAK can be induced by clustering of chimeric receptor proteins that possess integrin β1, β3 or β5 cytoplasmic domains and extracellular and transmembrane domains of the CD4 or interleukin-2 receptor molecules (Akiyama et al., 1994; Lukashev et al., 1994). This suggests that the β subunit cytoplasmic domains contain sequences responsible for stimulating FAK phosphorylation and the other regions of the integrin α and β integrin subunits are not required. Whether the β subunit activates

FAK by direct interaction or is physically linked to FAK through an intermediary protein or, possibly, activates an unidentified enzyme, which then activates FAK, has not been defined. However, it seems that the β subunit cytoplasmic domain is important in triggering FAK phosphorylation through integrins.

Some evidence that direct binding of FAK to the β subunit is not required for phosphorylation comes from studies which defined specific sequences within the β3 subunit cytoplasmic domain which were necessary to initiate FAK phosphorylation (Tahiliani et al., 1997). Amino acids in both the membrane-proximal and C-terminal ends of the cytoplasmic domain were required to signal FAK phosphorylation, but a region of the β3 subunit cytoplasmic domain identified as the putative FAK binding site was not necessary for FAK phosphorylation to occur (Schaller et al., 1995). It has also been shown that recruitment of FAK to a submembraneous localisation, independently of integrins, is sufficient to cause phosphorylation of FAK. Inducing membrane association of FAK, by combining full length FAK with the extracellular and transmembrane regions of the CD2 receptor protein, led to its increased phosphorylation and kinase activity in both adherent and suspended cells. Membrane association of FAK appears to result in its activation and phosphorylation, possibly mimicking the events that occur during FAK localisation to focal adhesions upon integrin engagement. However, induced membrane association of FAK, unlike the specific localisation of FAK to focal adhesions during adhesion through integrins, may allow interaction between FAK and other PTK's that induce unregulated phosphorylation of FAK (Chan et al., 1994).

It has been noted that adhesion through the $\alpha_{IIb}\beta_3$ integrin alone is not sufficient to stimulate platelet spreading and phosphorylation of FAK. In addition to adhesion through $\alpha_{IIb}\beta_3$, stimulation by an exogenous agonist, such as adrenaline or thrombin is also required for phosphorylation of FAK to occur (Lipfert et al., 1992; Shattil et al., 1994). This additional stimulation may be required to change the activation state of the integrin (Pelletier et al., 1995). The activation state of integrins may be important in regulating the "outside-in" signaling that leads to FAK activation.

Studies in platelets also provide evidence that in some cases FAK may be downstream of other kinases, one of which may be PKC, that are activated after integrin

engagement (Huang et al., 1993; Haimovich et al., 1996). In Chinese hamster ovary cells activation of PKC is required for subsequent FAK activation. PKC acts on FAK indirectly however, possibly through effects on the cytoskeleton (Vuori and Ruoslahti 1993). Evidence that there may be integrin activated signals upstream of FAK such as activation of PKC or ion channels (Bianchi et al., 1995) may reflect integrin specific signaling pathways that allow activation of FAK by different pathways from different integrins.

Although FAK was identified as a possible mediator of integrin signaling by virtue of its increased phosphorylation and activation by integrin adhesion, there is also evidence that FAK can be dephosphorylated as a result of integrin adhesion. Stimulation of murine thymocytes with specific anti-integrin antibodies resulted in the dephosphorylation of FAK (Kanazawa et al., 1995). The same antibodies stimulated FAK phosphorylation in fibroblast cells suggesting integrin induced dephosphorylation of FAK was specific to thymocytes. This is the only reported case of integrin-induced dephosphorylation of FAK and may be a reflection of different adhesive or migratory behavior in thymocytes.

It is still not clear exactly how FAK becomes activated after integrin adhesion but from the evidence presented above it seems that there may not be a single answer that is universal in integrin signaling. It is known that there is some redundancy in integrin-ligand interactions, with more than one type of integrin able to bind a single substratum. If different integrins are able to activate different signaling pathways, including differential regulation of FAK, this may be one reason for this apparent redundancy, allowing cells to regulate signaling by expressing specific integrins.

Activation of FAK by non-integrin signaling

Several different types of receptor, other than integrins, are now known to initiate signaling cascades that result in activation and phosphorylation of FAK. This has lead to speculation that FAK is involved in many different signaling pathways and is perhaps a point of convergence between these pathways, allowing cross-talk between them (reviewed by Zachary and Rozengurt 1992; Rozengurt 1995). The types of receptors that stimulate FAK phosphorylation and what is known about the pathways through which they act on FAK is discussed below. Many of the receptor agonists mentioned here are known to cause increased membrane ruffling and increased cell migration, both

phenotypes associated with changes in adhesion and the actin cytoskeleton. FAK may represent a point at which these agonists can affect integrin signaling pathways and induce changes in cell adhesion and motility.

Receptor tyrosine kinases (RTK's)

RTK's posses intrinsic kinase activity that, once they are activated, allows them to phosphorylate substrate proteins. Agonists of tyrosine kinase receptors that stimulate an increase in phosphorylation of FAK include fibroblast growth factor (FGF; Hatai et al., 1994) hepatocyte growth factor/scatter factor (Matsumoto et al., 1994), platelet-derived growth factor (PDGF; Rankin and Rozengurt 1994), vascular endothelial growth factor (Abedi and Zachary 1995) and insulin-like growth factor-1 (IGF-1; Leventhal et al., 1997).

A common result of agonist binding to RTK's is autophosphorylation of the receptor. The autophosphorylation site provides a binding site for molecules containing SH2 domains, allowing them to associate with, and be phosphorylated by, the receptor. The fact that FAK does not posses SH2 domains means it is unlikely to be phosphorylated by RTK's through direct interaction. It is therefore likely that receptor binding results in activation of a second messenger that is responsible for the increased FAK phosphorylation.

Some studies suggest the presence of an intact microfilament network is important for phosphorylation of FAK, similar to results seen for G protein-coupled receptors (see below). Stimulation of Swiss 3T3 cells with low concentrations of PDGF results in phosphorylation of FAK. In contrast, higher concentrations of PDGF, that disrupt the cytoskeleton, do not increase FAK phosphorylation (Rankin and Rozengurt 1994). PDGF possibly mediates its effect on FAK through changes in the cytoskeleton mediated by phosphatidylinositol (PI)-3 kinase activation of the small G protein Rac (Chen and Guan 1994; Rankin et al., 1996). An interesting effect of FGF on BALB/c 3T3 cells is that FAK becomes phosphorylated on serine residues, suggesting a serine kinase is activated downstream of the FGF receptor (Hatai et al., 1994). It will be of interest to determine if the FAK serine residues phosphorylated in response to FGF are identical to the serine residues phosphorylated on FRNK. The serine kinase PKA has been implicated in

phosphorylation of FRNK and may therefore be the kinase activated by FGF receptors to induce serine phosphorylation of FAK. The serine phosphorylation of FAK may actually result in reduced signaling through FAK due to the disruption of FAK signaling complexes (Yamakita et al., 1999).

One of the few cases where FAK has been observed to be specifically dephosphorylated is in response to insulin (Knight et al., 1995; Pillay et al., 1995). Insulin may dephosphorylate FAK indirectly through inhibition of Src activity (Tobe et al., 1996). IGF-1 has been shown to stimulate phosphorylation (Leventhal et al., 1997), and dephosphorylation of FAK (Konstantopoulos and Clark 1996). However, IGF-1 only has the effect of dephosphorylating FAK in cells expressing high levels of the insulin receptor and it was suggested that hybrid insulin/IGF-1 receptors form in these cells. IGF-1 is thought to act through these receptors, with the C-terminus of the insulin receptor being important in signaling for FAK dephosphorylation.

Insights into RTK signaling to FAK have shown that different second messengers may be important for different receptors. This may reflect different signaling pathways used by different receptors as well as cell type specific signaling pathways. FAK may allow regulation between growth/mitogenic effects of these receptors and their effects on adhesion and motility.

G protein-coupled receptors

Numerous agonists of G protein-coupled receptors have been shown to stimulate an increase in the phosphotyrosine level of FAK. Many of these agonists are small mitogenic neuropeptides such as bombesin (Sinnett-Smith et al., 1993; Charlesworth et al., 1996), vasopressin (Zachary et al., 1992), endothelin-1 (Saville et al., 1994; Haneda et al., 1995), bradykinin (Leeb-lundberg et al., 1994; Lee and Villereal 1996), angiotensin II (Earp et al., 1995; Okuda et al., 1995) and cholecystokinin-8 (Taniguchi et al., 1994). Other agonists include lysophosphatidic acid (LPA; Kumagai et al., 1993; Chrzanowska-Wodnicka and Burridge 1994), platelet-activating factor (Soldi et al., 1996) and agonists of muscarinic cholinergic receptors (Gutkind and Robbins 1992; Linseman et al., 1998).

G protein-coupled receptors have no intrinsic kinase activity and are generally associated with the activation of serine/threonine protein kinases, such as protein kinase C

(PKC). The activation of FAK links G protein-coupled receptors to new signaling pathways through tyrosine kinases. Stimulation of G protein-coupled receptors can lead to the activation of many different second messenger signaling pathways and the exact mechanism of FAK phosphorylation by these receptors is not known. There are, however, some similarities in the results of the studies mentioned above that point to common pathways in the phosphorylation of FAK by activation of these receptors.

Activation of many G protein-coupled receptors leads to activation of phospholipase C (PLC) which, subsequently, leads to elevated levels of intracellular calcium (Ca^{2+}) and activation of PKC. Several of the studies above show little effect of Ca^{2+} levels on FAK phosphorylation but an increased FAK phosphotyrosine level after direct activation of PKC by phorbol esters, suggesting PKC involvement in FAK phosphorylation through G protein-coupled receptors. FAK has been shown to have a consensus sequence for PKC phosphorylation, supporting this possibility (Schaller et al., 1992). However, inhibition of PKC was shown to only partially prevent phosphorylation of FAK by bradykinin, endothelin and LPA. PKC may, therefore, only be partially responsible for increased FAK phosphorylation, acting in synergy with other pathways activated by the G-protein coupled receptors.

Another feature important to FAK phosphorylation by many of these agonists is the requirement for an intact cytoskeleton. This requirement may reflect another possible pathway of FAK phosphorylation, through Rho mediated cytoskeletal organisation. LPA has been shown to activate Rho, a member of the Ras superfamily of G proteins, leading to changes in the actin cytoskeleton (Zhang et al., 1997). These changes in the cytoskeleton may lead to relocation of FAK or an upstream activator of FAK resulting in FAK phosphorylation. Rho may also be important in signaling through other agonists as inhibition of Rho, by ADP-ribosylation using Clostridium botulinum C3 exoenzyme, inhibits endothelin and bombesin-stimulated phosphorylation of FAK (Rankin et al., 1994).

The possibility that different agonists stimulate FAK phosphorylation through different signaling pathways exists because of the number of different pathways activated by G protein-coupled receptors. Current studies point to involvement of PKC and cytoskeletal organisation in FAK phosphorylation through G protein-coupled receptors and the likely possibility that individual agonists lead to multiple signals that converge on FAK.

Oncogenic transformation

Cells that have undergone oncogenic transformation often become migratory and obtain the ability to proliferate without adhering to the ECM, a condition that initiates a type of programmed cell death, termed anoikis, in normal cells (reviewed by Ruoslahti and Reed 1994). The involvement of integrin signaling and FAK in these processes has recently become evident, suggesting FAK may have a role in tumour progression to a metastatic or proliferatory phase (for reviews see Juliano 1994; Akiyama et al., 1995; Malik and Parsons 1996).

Evidence for involvement of FAK in cell transformation comes from studies showing FAK to be phosphorylated in cells transformed by v-Src (Schaller et al., 1992; Guan and Shalloway 1992) and $p120^{BCR\text{-}ABL}$ (Gotoh et al., 1995; Salgia et al., 1995) as well as studies showing overexpression of FAK mRNA (Weiner et al., 1993), protein (Owens et al., 1995; Brunton et al., 1997) and changes in FAK phosphorylation (Scott and Liang 1995; Withers et al., 1996) in certain tumour cell types. The oncogenic PTK, v-Src, associates directly with FAK suggesting it may regulate FAK in Src-transformed cells and phosphorylation of FAK by v-Src may actually lead to degradation of FAK (Cobb et al., 1994; Fincham et al., 1995). The Src-FAK doublet may form a bipartite signaling complex that regulates many facets of cellular transformation by Src (reviewed in Parsons and Parsons 1997).

It has been suggested that oncogenic phosphorylation of FAK mimics integrin-stimulated phosphorylated of FAK, providing a stimulus only present in adherent conditions in normal cells. It follows that, if FAK provides a signal that suppresses programmed cell death pathways, as some reports suggest (Sonoda et al., 1997), phosphorylation of FAK may allow transformed cells to evade programmed cell death

once they lose adherence to the ECM. Regulation of FAK by oncogenic transformation may also provide a means by which transformed cells can regulate adhesion and cytoskeletal assembly during metastatic or invasive stages that require changes in motility and cell shape. A better understanding of the function of FAK in normal cells will allow insight into the role of FAK in tumor progression.

Other activators of FAK

There are several studies that show the variety of stimuli that can induce FAK phosphorylation encompasses many other areas than those mentioned above. Relatively little is known about the mechanism of their action so they will just be briefly mentioned here to give an idea of the fields under study. Some of the agonists include sphingosylphosphorylcholine (Seufferlein and Rozengurt 1995), Aβ peptides (Zhang et al., 1994), prolactin (Canbay et al., 1997), fluid shear stress (Hamasaki et al., 1995) and engagement of the T cell receptor (Berg and Ostergaard 1997). There is also evidence that the second messengers sphingomyelinase (Sasaki et al., 1996), sphingosine 1-phosphate (Wang et al., 1997) can also cause an increase in FAK phosphorylation, possibly linking different signaling pathways to those signaling through FAK.

FAK-related proteins

Since the identification of FAK as a new type of cytoplasmic tyrosine kinase several other proteins have been cloned that make up the FAK subfamily. All of these proteins have been identified in the last few years and relatively little is known about their regulation and function within the cell. The high degree of homology between these proteins and FAK raises the possibility that there may be some redundancy between their functions.

Proline-rich tyrosine kinase 2

The FAK subfamily was expanded with the identification of an FAK related protein, termed proline-rich tyrosine kinase 2 (Pyk2), in human tissue (Lev et al., 1995). Three other PTK's were identified independently and later found to be identical to Pyk2. The three other PTK's were named cell adhesion kinase β (CAKβ; Sasaki et al., 1995) related adhesion focal tyrosine kinase (RAFTK; Avraham et al., 1995) and calcium-

dependent tyrosine kinase (CADTK; Yu et al., 1996). Another possible FAK related gene, identified by low-stringency screening of a hippocampus cDNA library, was localised to chromosome 8p11.2-p22 and designated as FAK2 (Herzog et al., 1996). A further study localising the Pyk2 gene on chromosome 8p21.1 showed remarkably similar results to the study localising the FAK2 gene, suggesting FAK2 may also turn out to be identical to Pyk2 (Inazawa et al., 1996).

These studies showed Pyk2 to be a 123kDa protein with a similar overall structure to FAK, exhibiting about 45% identity at the amino acid level. Like FAK, Pyk2 has a central catalytic domain flanked by large N and C-terminal non-catalytic domains (Figure 1). The major autophosphorylation site of FAK on tyrosine 397 is conserved in Pyk2, as are the proline rich sequences in the C-terminal domain. The FAT sequence in FAK and the corresponding region of the Pyk2 C-terminal domain show a high homology, suggesting a similar function in localising Pyk2 to a subcellular compartment.

Initially, several differences were identified between Pyk2 and FAK signaling. Pyk2 was shown to be localised to regions of cell to cell contact, rather than in focal adhesions, and the phosphorylation of Pyk2 was unaffected by adhesion to a fibronectin substratum (Sasaki et al., 1995). Also, increases in intracellular Ca^{2+} levels from extracellular or intracellular stores were shown to be required for phosphorylation of Pyk2 to occur (Lev et al., 1995; Yu et al., 1996). More recent studies have also shown that there are differences in the regulation of Pyk2 and FAK, not only in the stimuli that activate them but also the pathways involved in their activation (Siciliano et al., 1996).

In contrast to initial studies, recent evidence suggests that Pyk2 does have many similarities to FAK. It has been shown that Pyk2 becomes tyrosine-phosphorylated in response to integrin adhesion (Li et al., 1996; Astier et al., 1997a; Gismondi et al., 1997). Also, Pyk2 associates with paxillin and other signaling molecules such as Src and Grb2 (Ganju et al., 1997; Hiregowdara et al., 1997). Involvement of Pyk2 in various signaling pathways is discussed below.

FAKB

A second FAK related protein was identified in T and B-lymphocytes and was designated, FAKB (Kanner et al., 1994). It was reported that FAKB was differentially regulated from FAK and formed stable complexes with ZAP-70, another PTK. Stimulation of FAKB phosphorylation was noted after engagement of the T and B cell antigen receptors, a stimulus that has also been observed to induce phosphorylation of FAK (Berg and Ostergaard 1997). FAKB has also been shown to respond to β2 integrin engagement in the same cell type (Kanner 1996). The structure of FAKB is currently unknown.

The FAK subfamily appears to consist of PTK's with very similar attributes that may be able to cover for a missing member of the family due to some redundancy between them. Each member also show's some specific differences from the others that presumably distinguishes its role within the cell.

CELL ADHESION AND INTRACELLULAR SIGNALING

Cells adhere to one another and to the ECM through specialised adhesion sites on the cell surface. These adhesion sites include adherens junctions (Yap et al., 1997), desmosomes and hemidesmosomes (Green and Jones 1996), tight junctions (Takeda and Tsukita 1995; Kim 1995) and gap junctions (Yeager and Nicholson 1996). They differ in their molecular composition and type of adhesion molecule found at the site (Joseph-Silverstein and Silverstein 1998). An important function of these adhesion sites is the transduction of signals into the cell (Rosales et al., 1995; Lampugnani and Dejana 1997). The following section will focus on the integrin family of adhesion molecules and their involvement in adhesion formation and signal transduction with emphasis on the involvement of FAK.

Integrins and intracellular signaling

The integrin family of cell adhesion receptors are transmembrane heterodimers consisting of two transmembrane subunits, α and β. Currently, 16 α and 8 β subunits have been identified and they combine to form 22 known heterodimers (Humphries and Newham 1998). They are the main class of ECM-binding receptor and can transduce signals in both directions across the plasma membrane, referred to as 'inside-out' and

'outside-in' signaling (Garratt and Humphries 1995; Dedhar and Hannigan 1996). Integrins are known to influence many different signaling pathways including intracellular pH and calcium levels, tyrosine phosphorylation and lipid metabolism (reviewed in Schwartz 1994). There are several cytosolic molecules with which integrins associate that are involved in mediating integrin signals, such as FAK (Guan 1997), integrin-linked kinase (Hannigan et al., 1996) and β_3-Endonexin (Shattil et al., 1995).

Inside-out signaling

Inside-out signaling involves changes in adhesion between integrin and ligand as a result of changes in the cytoplasmic domains of the integrin (Puzon-McLaughlin et al., 1996; Kolanus and Zeitlmann 1998). The mechanisms of integrin signaling are not presently clear but studies suggest that the α subunit cytoplasmic domain may interact with the β subunit cytoplasmic domain to maintain the integrin in a low affinity state. Disruption of this interaction may lead to 'activation' of the integrin (Hughes et al., 1996). Studies also support the hypothesis that the α subunit acts as a regulator of integrin function by restricting the interactions of cytoskeletal and signaling molecules with the β subunit (Leong et al., 1995; Briesewitz et al., 1995). Changes in the cytoplasmic domains are thought to induce conformational changes in the extracellular domains that may expose ligand binding sites and change the adhesive properties of the integrin (Faull and Ginsberg 1996; Sugimori et al., 1997).

Regulation of integrin activity is, in some cases, associated with phosphorylation of either the α or β cytoplasmic domains (Gimond et al., 1995; Takagi and Saito 1995; Van Willigen et al., 1996). However, phosphorylation may be a result of integrin activation rather than an inducer of activation, and studies suggest it may be associated with subcellular localisation of integrins (Barreuther and Grabel 1996) or regulating integrin association with cytoplasmic signaling molecules (Law et al., 1996).

Outside-in signaling

In the same way that cytoplasmic signals can affect the extracellular conformation of the integrin, ligand binding to an integrin can initiate signaling cascades within the cell through outside-in signaling (Meredith et al., 1996). Integrins bind to one or more specific

amino acid sequences within their ligands (Danen et al., 1995; Ruoslahti 1996). Ligand is bound by specific domains on both the α and β subunits in a divalent cation dependent manner (Hogg et al., 1994; Loftus et al., 1994). Ligand binding may induce conformational changes in the integrin that allow transduction of signals into the cell (Humphries 1996; LaFlamme et al., 1997; Loftus and Liddington 1997). This may result in phosphorylation of the cytoplasmic domains allowing integrins to form signaling complexes with SH2 domain containing proteins (Law et al., 1996). Integrin signaling may be regulated by divalent cations influencing ligand dissociation or possibly by proteolytic cleavage of the cytoplasmic domains (Smith 1997).

The cytoskeleton and focal adhesions

In cells grown *in vitro*, integrins localise to specific sites of adhesion termed focal adhesions. Focal adhesions were first identified using interference reflection microscopy, a technique that allows visualisation of the separation between the ventral cell membranes and the substratum of cultured cells (Verschueren 1985). Focal adhesions are small regions of the cell membrane (10μm in length and 0.5μm in width), representing regions of extremely close association between the membrane and substratum. The separation between the membrane and substratum is approximately 10-15nm in focal adhesions, compared to 100nm in non-adherent regions (Heath and Dunn 1978; Chen and Singer 1982).

In terms of molecular structure, focal adhesions are multi-protein complexes linking integrins to the cytoskeleton and intracellular signaling pathways (Jockusch et al., 1995; Yamada and Geiger 1997). Certain cytoskeletal proteins such as α-actinin (Pavalko and LaRoche 1993), vinculin (Jockusch and Rüdiger 1996), talin (Tranqui and Block 1995) and paxillin (Cattelino et al., 1997) have been shown to localise to focal adhesions, along with many other proteins (Figure 2). These proteins bind to each other in certain combinations and since some of the proteins are known to bind actin and others to integrins, they form a structural link between integrins and actin microfilaments (Hemmings et al., 1995; Simon and Burridge 1994; Taylor et al., 1998b).

Paxillin is one of several proteins found in the focal adhesion that are classed as adaptor or docking proteins due to the fact they have no enzyme activity but instead

contain several motifs within their amino acid structure that function as protein binding domains. Paxillin contains one cysteine-rich sequence known as a LIM domain and three LIM-like sequences. Paxillin also contains five tyrosine-containing sequences that may be SH2 binding sites and a short proline-rich region that may be an SH3 binding site (Turner and Miller 1994). So, paxillin contains multiple sequences for protein interaction allowing it to associate with many other proteins. Tyrosine phosphorylation of paxillin occurs upon cell adhesion and appears to be coordinated with increased tyrosine phosphorylation of FAK (Burridge et al., 1992). This, along with the fact that paxillin has an FAK binding site (Turner and Miller 1994), has implicated paxillin as a possible substrate for FAK. Tyrosine phosphorylation of paxillin by FAK and Src may be important in the regulation of the cytoskeleton after adhesion allowing cell spreading and possibly cell migration to occur (Richardson et al., 1997a).

Although, the exact composition and precise molecular interactions in a focal adhesion remain unknown, scanning force microscopy reveals changes in focal adhesion structure over time (Dunlap et al., 1996), and cells with null-mutations in focal adhesion proteins are still able to form focal adhesions (Volberg et al., 1995). Also, actin has also been revealed to bind directly to certain integrin subunits (Kieffer et al., 1995), suggesting there is more than one possible model for a focal adhesion and that they may actually change or mature over time.

Focal adhesions link integrins to actin microfilaments that form 'stress fibres', part of the contractile cytoskeleton (Katoh et al., 1998). The link that focal adhesion proteins provide between integrins and the cytoskeleton may allow the cytoskeleton to form a physical framework, connected to the ECM through integrins, that enables cells to detect mechanical stresses put on them by their environment, a model termed 'tensegrity' (Ingber 1997; Maniotis et al., 1997).

Since tyrosine phosphorylation is required for focal adhesion formation (Burridge et al., 1992; Ridley and Hall 1994; Retta et al., 1996), and many of the focal adhesion components are tyrosine phosphorylated (Bockholt and Burridge 1993), it was originally thought that tyrosine phosphorylation of structural components of the focal adhesion enabled protein interaction and focal adhesion formation. However, recent studies have

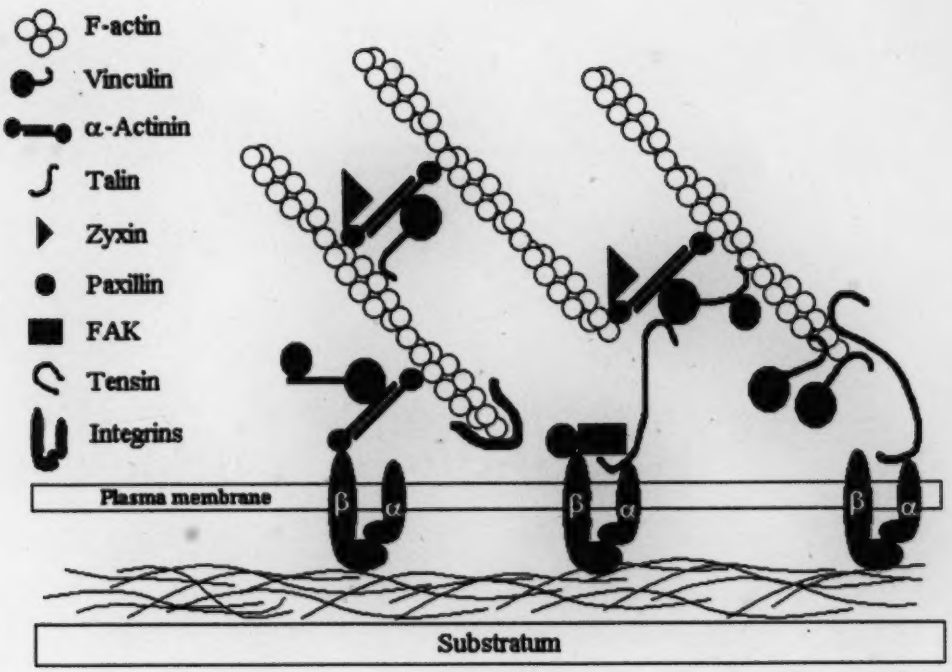

Figure 2. A model of a focal adhesion. The model shows possible protein interactions that may occur within a focal adhesion. Adapted from Jockush and Rüdiger (1996).

shown that some of the proteins known to be tyrosine phosphorylated in focal adhesions do not require phosphorylation in order to localise to focal adhesions (Bellis et al., 1995; Brown et al., 1998). Also, rearrangement of focal adhesion proteins can occur in the absence of new tyrosine phosphorylation (Silletti et al., 1996). Interestingly, the tyrosine kinase activity needed for focal adhesion formation has recently been demonstrated to be separate from the tyrosine kinase activity that causes phosphorylation 'within' focal adhesions and specifically separated from FAK activity (Schneider et al., 1998). So, it seems that tyrosine kinase activity is required for focal adhesion formation, but it occurs upstream of focal adhesion formation and not within the focal adhesion itself, and FAK is not involved.

Other evidence also suggests FAK is not involved in focal adhesion formation. Cells from FAK deficient mouse embryos actually form more focal adhesions than normal mouse embryo cells in culture (Ilic et al., 1995a). Also, mouse aortic smooth muscle cells have been shown to form focal adhesions in the absence of FAK activity (Wilson et al., 1995). Further evidence comes from studies showing that integrins can associate with actin in situations where FAK is not activated or not present (Katoh et al., 1995; Defilippi et al., 1997). Mutated integrin subunits that are unable to form focal adhesions still activate FAK, further supporting the fact that FAK is not responsible for focal adhesion formation (Lyman et al., 1997).

So how are focal adhesions formed? A possible explanation lies in the small GTPase, Rho. It was demonstrated that Rho is an important second messenger involved in focal adhesion and stress fibre formation induced by growth factors (Ridley and Hall 1992), and that integrin adhesion was not sufficient to form focal adhesions in the absence of Rho activity (Hotchin and Hall 1995; Barry et al., 1997). Stimulation of Rho activity has also been correlated with increased tyrosine phosphorylation of FAK and paxillin (Flinn and Ridley 1996; Lacerda et al., 1997).

There are several downstream effectors of Rho activity that may mediate Rho's effect on the cytoskeleton (Hall 1994; Machesky and Hall 1996; Hall 1998). Rho has been shown to cause stress fibre formation through bundling of actin filaments (Machesky and Hall 1997) and this may be achieved through Rho activated kinases which phosphorylate

myosin phosphatase (Amano et al., 1997; Ishizaki et al., 1997). The phosphorylation of myosin phosphatase inactivates it resulting in an increase in phosphorylation of its substrate, the myosin light chain. Phosphorylation of the myosin light chain facilitates its interaction with actin, leading to cross-linking of actin filaments (Kimura et al., 1996).

These facts have lead to the theory that Rho induces myosin light chain phosphorylation, and the subsequent actin/myosin bundling and generation of tension results in stress fibre formation. Integrins associated with the ends of these stress fibres are clustered together by the bundling of the fibres leading to focal adhesion formation (Burridge and Chrzanowska-Wodnicka 1996). The fact that focal adhesions may be formed by the bundling of actin fibres rather than vice-versa is supported by studies showing actin cross-linking proteins can restore focal adhesions in cells lacking focal adhesions because they are grown under serum free conditions (Manenti et al., 1997).

It has been observed that differences in the cytoskeleton and focal adhesion formation occur when cells are grown in 3-dimensional or on flexible substrata (Corbett et al., 1996; Pelham and Wang 1997). Cells respond to changes in the rigidity of their substratum by changing the strength of adhesive sites and focal adhesion proteins are important in the regulation of that adhesive strength (Girard and Nerem 1995; Choquet et al 1997; Ezzell et al., 1997). Focal adhesions may form in cultured cells as a result of the extremely rigid surface on which the cells are grown. Contraction of stress fibres, resulting in isometric tension within the cytoskeleton because of the rigid substratum (Chrzanowska-Wodnicka and Burridge 1996), may lead to strengthening of adhesion sites by accumulation of focal adhesion proteins and eventual formation of focal adhesions, a situation that may only occur in specific situations *in vivo*, where high levels of stress are acting upon cells (discussed below).

Protein kinase C may be involved in focal adhesion formation (Tang et al., 1995). Integrin adhesion can activate specific PKC isoforms and activation of PKC has been shown to increase paxillin tyrosine phosphorylation (Wrenn and Herman 1995; Sakamoto et al., 1996; Haller et al., 1998). Also, PKC may act upstream of FAK to induce FAK expression or enhance FAK stability (Mogi et al., 1995; Kanan et al., 1997).

Disruption of focal adhesions through calpain-mediated proteolytic cleavage of

FAK (Cooray et al., 1996), and cytoskeletal proteins (Yamaguchi et al., 1994), as well as integrin cytoplasmic domains (Meredith et al., 1998), has been proposed to be a mechanism through which integrin mediated signaling is terminated. Certain tyrosine phosphatases, such as PTP1B, also localise to focal adhesions and may act to regulate signaling pathways through control of phosphorylation levels (Liu et al., 1998).

Signaling through FAK

If FAK is not involved in focal adhesion formation, what role does it play in integrin signaling? Once the focal adhesion is formed, FAK may be required for formation of enzymatic signaling complexes activated by adhesion. FAK could cause the increased phosphorylation observed within focal adhesions, allowing SH2 domain-mediated interactions between signaling molecules, such as Crk and Csk, and focal adhesion components (Bergman et al., 1995; Schaller and Parsons 1995), as well as directly binding to signaling molecules such as Src, Cas, PLC and PI 3-kinase (Guinebault et al., 1995; Harte et al., 1996; Carloni et al., 1997; Thomas et al., 1998). The formation of such signaling complexes could have an influence on different aspects of cell behavior (discussed below).

Regulation of cell motility

The actin cytoskeleton is important in formation of filopodia and lamellipodia during migration and for generating tension that breaks adhesions as the cells migrate (Jay et al., 1995; Mitchison and Cramer 1996). The ability of cells to regulate their surface adhesiveness and contraction of the cytoskeleton is important in regulating the levels of cell migration (Opas 1995). Since integrins are the main class of receptors for the ECM and they link to the cytoskeleton, it isn't suprising that regulation of integrin adhesion is important in controlling cell motility (reviewed in Huttenlocher et al., 1995). Increasing the ligand-binding affinity or cytoskeletal association of integrins can reduce cell motility (Huttenlocher et al., 1996; Palecek et al., 1997), suggesting an excess level of adhesion site formation negatively effects motility. Conversely, cytokines that stimulate motility cause a reduction in the number of focal adhesions seen in those cells (Dunlevy and Couchman 1995).

Factors that regulate the formation, disassembly or stability of cell adhesions are clearly important in controlling motility and there is a growing interest in the involvement of FAK in regulating cell motility through its role in adhesive signaling events. Lack of FAK expression has been shown to have a negative effect on cell motility (Ilic et al., 1995a; Ilic et al., 1996), as has displacement of FAK from focal adhesions (Gilmore and Romer 1996). Expression of FAK has also been correlated with increased migration in certain cell lines (Akasaka et al., 1995; Cary et al., 1996). In contrast to original theories suggesting FAK played a role in focal adhesion assembly, evidence is now suggesting that FAK is important in disassembly of focal adhesions and it is this role in stimulating the turnover of adhesions through which FAK may promote migration (Fincham and Frame 1998). Supporting the fact that disruption of focal adhesions increases migration of cells are studies showing cells that lack expression of vinculin, a structural component of focal adhesions, also have increased motility (Coll et al., 1995). Vinculin deficient cells not only have increased migration rates but also show increased levels of FAK phosphorylation (Xu et al., 1998).

How FAK signaling may regulate motility or focal adhesion turnover is unclear. The fact that the kinase activity of Src is also required to prevent large focal adhesions forming (Kaplan et al., 1994), and that Cas may be downstream of FAK in integrin mediated migration (Cary et al., 1998), suggests that recruitment of other signaling molecules is important. FAK associates with Graf, a GTPase activating protein for Rho (Taylor et al., 1998a). Through Graf, FAK may provide a negative feedback control that inhibits Rho activity, preventing focal adhesion formation and maintaining adhesion at a level that promotes motility.

Growth factors can cross-talk with integrin receptors and modulate integrin mediated cell motility (for review see Klemke et al., 1994). As many RTK's have been shown to activate FAK (see above), there is the possibility that FAK may be an important point of convergence through which growth factors can affect adhesion and migration.

The MAP kinase pathway

A kinase cascade regulated by the small G-protein Ras activates mitogen-activated protein kinases (MAPKs). Once activated, MAPKs translocate to the nucleus where they

regulate the activity of transcription factors (Treisman 1996). Adhesion to the ECM, treating cells with anti-integrin antibodies or mechanical stressing of integrins can result in activation of the MAPK cascade (Chen et al., 1994; Ishida et al., 1996; Schmidt et al., 1998 Wei et al., 1998). FAK was initially thought to be part of the link between integrins and the MAPK pathway after it was shown that FAK bound to growth factor receptor binding protein 2 (Grb2). Grb2 is an activator of son of sevenless (Sos) which exchanges GDP for GTP on Ras, activating the MAPK cascade (Schlaepfer et al., 1994). It has been suggested that integrin adhesion results in FAK autophosphorylation, this allows the SH2 domain of Src to bind to FAK, activating Src. Src then phosphorylates FAK on tyrosine 925 which provides a binding site for the SH2 domain of Grb2 and a possible link to the MAPK pathway (Schlaepfer et al., 1994). Support for FAK involvement in MAPK activation comes from experiments overexpressing FAK in human 293 epithelial cells, with a resulting increase in MAPK activity (Schlaepfer and Hunter 1997). Src activity was required for the effect to occur but binding of Grb2 to FAK was not, suggesting an alternative pathway to MAPK activation (Schlaepfer and Hunter 1997). In fact, there appear to be several possible downstream pathways to MAPKs from an FAK-Src complex through the Shc, Cas and Nck proteins (Schlaepfer et al., 1997). FAK and Src are also involved in phosphorylation of SHPS-1 leading to its association with SHP-2, a tyrosine phosphatase known to affect MAPK activity, providing another pathway from FAK activation to MAPK regulation (Tsuda et al., 1998).

Some studies clearly show FAK-independent activation of MAPK through integrin adhesion suggesting there are FAK-dependent and independent pathways leading to MAPK activation (Lin et al., 1997;Clark and Hynes 1996).

Although FAK can be activated by stimulation of G protein-coupled receptors and RTK's, it appears to have no function in the MAPK activation from these receptors (Seufferlein et al., 1996). Integrin signaling can cross-talk with growth factor signaling pathways to enhance MAPK activity in some cells (Miyamoto et al., 1996), although in other studies it has been noted that the kinetics of MAPK activation are different under stimulation by adhesion or growth factors suggesting that they have different pathways, of which FAK may only be involved in the adhesion stimulated pathway (Zhu and Assoian

1995). An explanation for the lack of FAK involvement in MAPK activation by growth factors may be in the proteins with which FAK interacts. In monocytes, macrophage colony-stimulating factor can induce the association of FAK with Grb2, suggesting a link to the MAPK pathway, but FAK-Grb2 associates with dynamin, a GTPase implicated in endocytosis, rather than Sos in these cells. This provides evidence that there are separate pathways from RTK's leading to activation of FAK and the MAPK pathway, although it does suggest a role for the interaction between FAK and Grb2 (Kharbanda et al., 1995).

Although FAK has been shown to have no effect on cellular differentiation *in vitro* or *in vivo* (Ilic et al., 1995b; Moszczynska and Opas 1993), FAK may act to influence non-transcriptional effects of the MAPK cascade as there is evidence that MAPK's have some cytoplasmic functions too. A negative feedback loop may exist in which integrin adhesion leads to activation of MAPKs which, in turn, inhibits further integrin activation (Hughes et al., 1997).

Apoptosis

Apoptosis, is a type of 'programmed cell death', where specific intracellular signaling cascades are activated that result in fragmentation of DNA and membrane blebbing, ultimately leading to elimination of the cell (Kerr et al., 1987). Apoptosis occurs during embryonic development and is important in morphogenetic modeling of the embryo (Coucouvanis and Martin 1995; Sanders and Wride 1995; Sanders et al., 1997). It has been suggested that cells may have a default pathway leading to apoptosis and stimulation by extracellular signals can prevent entry into this pathway (Ishizaki et al., 1995). Adhesion to the ECM has been shown to be one such signal that can regulate apoptosis (Aoshiba et al., 1997a; Aoshiba et al., 1997b; McGill et al., 1997). In epithelial cells, adhesion to the ECM is required to prevent the cells from undergoing 'anoikis', a type of apoptosis (Frisch and Francis 1994; Metcalfe and Streuli 1997). Integrin adhesion, in particular, may be important in regulating apoptosis (Scott et al., 1997; Judware et al., 1998; Matter et al., 1998). Recent insights into the role of integrins in regulating apoptosis (Ruoslahti and Reed 1994; Bates et al., 1995; Frisch and Ruoslahti 1997; Otey 1998) have created interest in the possible involvement of FAK in transducing a 'survival' signal from integrins.

There are several lines of evidence that suggest FAK is directly involved in regulating apoptosis. Inhibition of FAK expression, in tumor cell lines that have high levels of FAK expression, by treating the cells with antisense oligonucleotides to FAK has been shown to result in the cells losing attachment to the substratum and undergoing apoptosis (Xu et al., 1996). In the same study, however, normal fibroblasts that expressed low levels of FAK did not detach from the substratum and undergo apoptosis. In contrast, however, other studies have shown that inhibition of FAK signaling does result in apoptosis in normal cell types (Hungerford et al., 1996), and constitutively activated FAK can prevent epithelial cells from undergoing anoikis (Frisch et al., 1996). The fact that antisense oligonucleotide inhibition of FAK expression increases the susceptibility of T98G cells to apoptosis, induced by treatment with hydrogen peroxide, further supports a role for FAK in providing a signal that prevents entry into the apoptotic pathway (Sonoda et al., 1997).

Further evidence that FAK signaling is associated with apoptosis comes from studies showing activation of apoptotic pathways can result in down regulation of FAK kinase activity and caspase-mediated cleavage of signaling proteins (including FAK) as well as cytoskeletal proteins (Kyle et al., 1997; Wen et al., 1997; Levkau et al., 1998; Gervais et al., 1998; Marushige and Marushige 1998; Widmann et al., 1998). Caspases are a family of cysteine proteases, some of which are involved in the execution phase of apoptosis (Salvesen and Dixit 1997). FAK was observed to be cleaved prior to entry into apoptosis suggesting that cleavage of FAK, and presumably its inactivation, may induce apoptosis, rather than be a result of apoptosis (Crouch et al., 1996). Also, treatment of suspended cells, that lack stress fibres, with Fas or Apo-2L induced apoptosis, suggesting FAK does not signal to prevent apoptosis indirectly, through downstream effects on changes in the cytoskeleton which occur when the adherent cells lose contact with the ECM (Wen et al., 1997).

FAK may function in a similar manner to Rho in preventing apoptosis. Inactivation of Rho, like FAK, can induce apoptosis (Bobak et al., 1997), and this effect may be through Rho mediated regulation of bcl-2 (Gomez et al., 1997). The integrin $\alpha 5\beta 1$ has also been shown to prevent apoptosis by regulating the levels of bcl-2 in parallel to FAK phosphorylation (Zhang et al., 1995b; Fukai et al., 1998). So, both Rho and FAK have

effects on adhesion and the cytoskeleton but appear to affect apoptosis through other pathways, although the cytoskeleton is thought to have a positive role in the membrane 'blebbing' that occurs during apoptosis (Atencia et al., 1997).

Integrin adhesion activates the small GTPase cdc42, a member of the Rho family, which has been shown to activate PI 3-kinase (Clark et al., 1998). PI 3-kinase, in turn, can stimulate phosphorylation and activation of Akt, a serine/threonine kinase that has anti-apoptotic effects (Dimmeler et al., 1998). Since FAK can bind to PI 3-kinase (Bachelot et al., 1996), there is the possibility that integrins could activate Akt and prevent apoptosis through more than one pathway.

Signaling through Pyk2

There is growing interest in signal transduction through Pyk2. Phosphorylation of Pyk2 can be induced by adhesion through integrins (Ma et al., 1997), and Pyk2 binds to some of the same proteins that have been shown to associate with FAK, such as paxillin, Cas and Graf (Ohba et al., 1998; Ostergaard et al., 1998). There also appears to be some similarities between the signaling pathways regulated by FAK and Pyk2. Like FAK, Pyk2 phosphorylates Cas in co-operation with Src (Astier et al., 1997b), is activated by growth factor stimulation (Hatch et al., 1998), and exists in different isoforms, including a C-terminal fragment presumably equivalent to FRNK (Dikic et al., 1998; Xiong et al., 1998). Recent work has shown some level of redundancy appears to exist between FAK and Pyk2 but there are also some distinct differences in their downstream signaling pathways (Schaller and Sasaki 1997; Derkinderen et al., 1998).

Like FAK, Pyk2 has been implicated in activation of the MAPK cascade (Lev et al., 1995), and may function by recruiting Src, Grb2 and Shc proteins to form multiple pathways to MAPKs, in a similar manner to FAK (Lev et al., 1995; Dikic et al., 1996). However, unlike FAK, Pyk2 appears to be involved in the activation of MAPK from G protein-coupled receptors (Della Rocca et al., 1997; Soltoff et al., 1998), as well as in activation of the Jun kinase pathway (Tokiwa et al., 1996; Ganju et al., 1998)

It is interesting to note that unlike FAK, Pyk2 appears to induce apoptosis in cells in which it is overexpressed (Xiong and Parsons 1997). This suggests there are differences in the signaling pathways through which these two proteins signal, despite their similar structure.

CELL ADHESION AND EMBRYOGENESIS

Cell-to-cell and cell-to-ECM interactions enable cells to organise themselves into tissues and to undergo the morphogenetic events that are required to form those tissues during development (reviewed in Gumbiner 1996; Hata 1996). The following section reviews the first morphogenetic event that occurs in vertebrate embryos, gastrulation, and the involvement of cell adhesion in early development.

Early vertebrate embryogenesis

The early stages of development are important in setting up the dorso-ventral and cranio-caudal axis of the embryo and in organising the primary germ layers. The stages of gastrulation are vital for these events and although they may use different mechanisms to accomplish the task, all vertebrate embryos undergo gastrulation. During gastrulation there is mass cell migration and transformation that results in formation of the epiblast, mesoderm and endoderm, the primary germ layers, each of which is composed of a different cell type. The epiblast is a layer of polarised epithelial cells, the mesoderm consists of fibroblast-like mesenchymal cells and the endoderm is a layer of loosely associated, non-polarised endothelial cells. Gastrulation is important in correctly positioning cells, which at this stage of development are precursor cells of various tissues, setting up the adult body plan (Tam and Behringer 1997).

Gastrulation in the chick embryo

The relatively large size and ease of handling of early chick embryos has made them an ideal model for studying early stages of development. In the avian embryo, the early cleavage stages occur while the egg is still in the reproductive tract of the adult. When the egg is laid the embryo is already a multicellular disc, called the blastoderm, lying on top of the yolk sac (Romanoff 1960; Bellairs and Osmond 1998). As development continues the blastoderm forms a central, somewhat transparent, area called the area

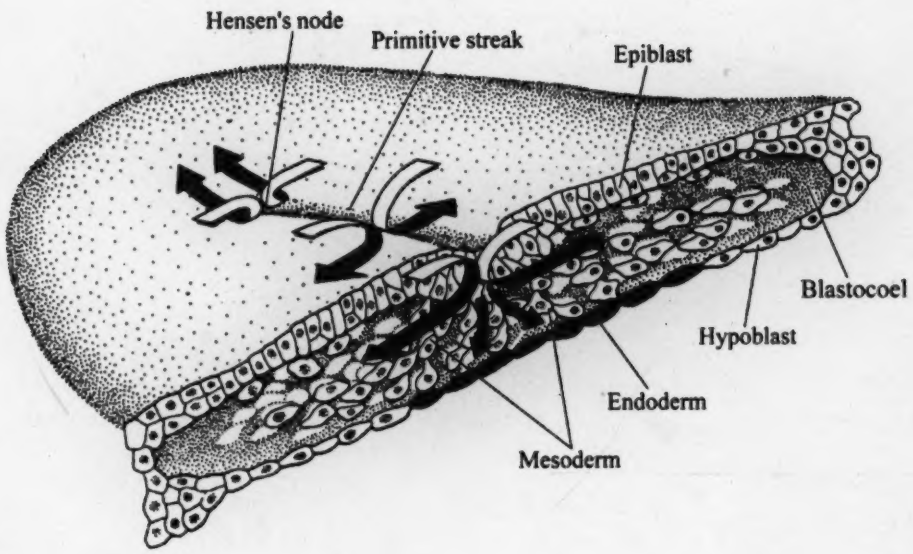

Figure 3. A diagrammatic representation of a gastrulating chicken embryo. Arrows represent the direction of cell movement as the epiblast ingresses through the primitive streak and mesoderm and endoderm migrate away. From Gilbert (1997).

pellucida which will form the embryonic tissues. This is surrounded by the area opaca, so called because of the opaque nature of the cells, which contain yolk droplets. The area opaca will form the extra-embryonic tissues. The area pellucida develops to form a primitive epithelial layer called the epiblast, which is above a loosely associated layer of cells termed the hypoblast. This two cell-layered embryo will become a three cell-layered embryo as it proceeds through the next step of development, the stage of gastrulation (Figure 3).

The process of gastrulation is induced when the hypoblast initiates the formation of the primitive streak in the overlying epiblast. Some of the epiblast cells develop an invasive phenotype before formation of the primitive streak (Sanders 1991; Toyoizumi et al., 1997) and these cells are thought to migrate and cluster together in the posterior of the embryo to begin formation of the primitive streak, under the influence of the hypoblast (Stern and Canning 1990; Toyoizumi and Takeuchi 1995). The primitive streak continues to form and extends anteriorly until it is approximately two thirds of the length of the area opaca. The most anterior part of the primitive streak forms a structure known as Hensen's node, the equivalent of the dorsal lip in the organiser of amphibian embryos (Bellairs and Osmond 1998). Formation of the primitive streak takes place between stages 2 to 4 of development and is fully formed after about 24 hours of development (Hamburger and Hamilton 1951). The cells of the epiblast migrate towards and ingress through the primitive streak (Figure 3). As they pass through the streak the epiblast cells transform into mesenchymal cells and migrate away from the streak, forming the mesoderm layer of the embryo. In order for the epiblast to ingress through the primitive streak it must first pass through the underlying basement membrane. It is believed that rather than enzymatically degrading the basement membrane below the primitive streak, the epiblast stops producing basement membrane constituents in the area of the streak and disruption of the basement membrane occurs (Sanders 1991). The mesoderm cells are thought to interact with and be influenced by the fibronectin rich ECM, present on the basal surface of the epiblast, as they migrate away from the primitive streak (Sanders 1982; Toyoizumi et al., 1991; Sanders et al., 1994). Not all the cells that ingress through the primitive streak contribute to the mesoderm layer, some cells displace the hypoblast layer and form a true endoderm layer (Sanders et al., 1978).

It has been shown that the fates of individual cells of the epiblast are somewhat predetermined. In mouse embryos it has been demonstrated that individual epiblast cells and their descendants will contribute to only one of the primary germ layers (Carey et al., 1995) and that mesoderm cells that have ingressed through the streak show pluripotency compared to the totipotent epiblast (Tam et al., 1997). Specific regions of the primitive streak give rise to mesoderm cells that contribute to particular structures and the mesoderm cells follow defined pathways depending on the structure for which they are destined (Schoenwolf et al., 1992; Psychoyos and Stern 1996). This suggests that mesoderm migration is directed, possibly either by interaction with the ECM or by chemotaxis.

There is evidence that certain growth factors, such as transforming growth factor β1 and activin, are involved in formation of the mesoderm in the chick embryo (Sanders and Prasad 1991; Stern et al., 1995). Several growth factors including FGF, PDGF and activin have been implicated in mesoderm formation in other species although exactly which growth factors are actually involved remains unclear (Ataliotis et al., 1995; Smith 1995; Ciruna et al., 1997). Growth factor effects on mesoderm induction have been correlated with activation of the MAPK cascade in *Xenopus* embryos (Umbhauer et al., 1995). It is likely that there are several factors that influence epithelial-to-mesenchymal transitions like those occurring at gastrulation with cross-talk between growth factor and adhesive signaling playing an important role (reviewed in Boyer et al., 1996; Birchmeier et al., 1995)

Integrins and regulation of morphogenesis

During early stages of development, morphogenesis involves extensive changes in cell adhesiveness as well as cell migration through the ECM (Sanders 1986; Brown and Sanders 1991). This provides an environment in which signaling pathways activated by the changing adhesive interactions may play an important role in regulating cell behavior and growth. Cell-to-cell interactions are important in morphogenesis (reviewed in Cunningham 1995; Marrs and Nelson 1996) but the ECM also provides cues that guide development. The ECM is made up of a variety of molecules that exist in different isoforms and splice variants, providing a means by which cells can receive position specific information based

on their local ECM composition through interaction with ECM receptors such as integrins (reviewed in Adams and Watt 1993). Integrins have been shown to have diverse roles in embryogenesis (reviewed in Lallier et al., 1994; Beauvais-Jouneau and Thiery 1997) and integrin expression is spatially and temporally regulated in early embryonic cells (Bronner-Fraser et al., 1992; Ramos et al., 1996). The regulation of both cadherin and integrin adhesion occurs during gastrulation in the mammalian embryo (Burdsal et al., 1993). Fibronectin is an important substratum in amphibian gastrulation (Wang et al., 1995; Ramos and DeSimone 1996) and mouse embryos lacking either fibronectin or the α5 subunit of the α5β1 fibronectin receptor show defects at late gastrulation stages (Georges-Labousse et al., 1996; Goh et al., 1997). Integrins are also important in the normal development of the *Drosophila* embryo (Martin-Bermudo et al., 1998). Further studies in *Drosophila* have revealed the importance of the Rho family members RhoA and Rac, and their ability to affect cell shape through the cytoskeleton, in development (Harden et al., 1995; Häcker and Perrimon 1998). The importance of the cytoskeleton in regulating cell shape is demonstrated in endothelial cells grown *in vitro* which can exert forces on the ECM through integrins and deform the ECM, resulting in formation of pathways which the cells use for migration (Davis and Camarillo 1995). It is possible that during development similar 'inside-out' signaling occurs to remodel the ECM through adhesive interactions.

As discussed above, integrin adhesion and subsequent signaling through FAK may regulate migration, differentiation or cell survival. There is also some correlation between FAK expression and cell proliferation, both *in vivo* and *in vitro*, although it appears that FAK expression is elevated as a result of the formation of new adhesions in the proliferating cells, rather than itself causing increased cell proliferation (Tremblay et al., 1996). There are a growing number of studies examining the expression and localisation of FAK during development and this information should provide a better understanding of the involvement of FAK in morphogenetic processes.

FAK expression in embryonic tissues

The results of several studies examining the levels of FAK expression and phosphorylation in a developmental context suggest a role for FAK during early

embryogenesis. The chick embryo shows a relatively constant expression of FAK, but changing levels of FAK tyrosine phosphorylation during early development up to day 8 (Turner et al., 1993). This is followed by declining levels of FAK, suggesting that FAK is most highly expressed and active during early remodeling of the embryo (Turner et al., 1993).

Tyrosine phosphorylated FAK is also expressed widely in the amphibian embryo, from cleavage stages onwards, becoming elevated at gastrulation (Hens and DeSimone 1995) together with FAK mRNA levels (Zhang et al., 1995a).

A significant role for FAK in development is suggested by studies on FAK-deficient mouse embryos which showed phenotypic defects by 8 days *post coitum*, in the late stages of gastrulation (Furuta et al., 1995). FAK-deficient mouse embryos showed head and lateral mesoderm formation along with initial heart and vasculature development, but further development did not occur. The deficits in the embryos suggested a general defect in the migration of mesodermal cells, similar to that observed in fibronectin and $\alpha 5$ integrin-deficient embryos (Georges-Labousse et al., 1996; Goh et al., 1997), and thus a role for FAK in regulating cell migration. These results are consistent with other studies in mouse embryos showing that FAK mRNA and protein levels increase from 7.5 days *post coitum* during development, suggesting that FAK is important during these early stages (Polte et al., 1994). As in the chick embryo, FAK protein is expressed throughout the mouse embryo, declining to low levels in the adult (Polte et al., 1994).

Focal adhesion kinase may also have a function in signaling from the ECM in cell migration and growth cone guidance in the developing brain. Examination of FAK expression during development of the rat brain reveals fairly constant levels of FAK during the embryonic period and decreased expression in the adult brain, although immunohistochemistry shows that all areas of the adult brain still express FAK. The phosphotyrosine level of FAK, however, is high in embryonic rat brain and decreases in postembryonic life (Burgaya et al., 1995; Serpente et al., 1996), supporting a role for FAK in development of neuronal tissues.

Analysis of FAK expression in developing human embryos shows FAK to be expressed in many tissues, but high levels of expression are found particularly in some

epithelia (Tani et al., 1996). An interesting result of the studies on human tissues is the localisation of FAK to the smooth muscle cells of the developing vasculature, a feature also seen in the later stages of vasculature development in the mouse embryo, where both smooth muscle cells and endothelial cells show high FAK levels (Polte et al., 1994). A possible explanation for this localisation was suggested to be the maintenance of tissue architecture in the developing vessels as they experience the forces exerted by the pressure of the blood.

FAK localisation in embryonic tissues

In cells grown in culture, adhesion to the underlying two-dimensional substratum results in the formation of focal adhesions (see above). It has been argued that these focal adhesions are formed as a result of the stresses experienced by cells growing on an inflexible two-dimensional substratum and are therefore not formed by cells *in vivo*, which experience deformable three-dimensional substrata (Burridge and Chrzanowska-Wodnicka 1996). This raises the question of whether events that occur at focal adhesions *in vitro* occur at all *in vivo*. Do the same cytoskeletal proteins such as tensin and vinculin link integrins to the cytoskeleton *in vivo*? Do signaling proteins, such as FAK, associate with integrins *in vivo*, and if not, is FAK activated by integrins through the same mechanism that occurs in focal adhesions? Also, is FAK involved in the same signaling pathways *in vivo* as those shown to be affected by FAK *in vitro*? Some of these questions are just starting to be answered as FAK is studied in a developmental context to determine its potential role in the embryo.

Studies in the chick embryo show that both the expression and phosphorylation of FAK are regulated during development (Turner et al., 1993), suggesting an active role in signaling pathways. There is also evidence that FAK may be associated with, and regulated by, Src family kinases *in vivo* (Grant et al., 1995; Serpente et al., 1996), and that it signals through phosphorylation of paxillin and interaction with Grb2 (Turner et al., 1993; Schlaepfer and Hunter 1996; Grant et al., 1995). There are, therefore, similarities between the molecular interactions observed *in vivo* and those observed *in vitro*. So, in embryonic cells, FAK may be involved in signaling pathways similar to those seen in cultured cells, but is FAK localised to specific sites of cell adhesion within the embryo?

Focal adhesion kinase appears to be expressed by most tissues *in vivo*, although there are only a small number of studies showing the subcellular localisation of FAK. One structure to which FAK has been localised *in vivo* is the myotendinous junction (Baker et al., 1994). The myotendinous junction (MTJ) is a site of cell-to-ECM adhesion that is associated with force transduction, and has been suggested to be analogous to a focal adhesion because focal adhesion proteins have been localised to the MTJ (Turner et al., 1991). Overexpression of FAK in artificially injured adult chicken tendon resulted in the accumulation of ECM at the injury site, suggesting that FAK is involved with the organisation of the ECM at these sites (Lou et al., 1997). The localisation of FAK to the MTJ supports the hypothesis that FAK is involved in signal transduction from sites of cell-to-ECM adhesion, and is localised to those sites. There is, however, evidence to suggest that, during development, FAK is not only associated with sites of cell-to-ECM adhesion, but also with sites of cell-to-cell adhesion in some epithelia.

One of the characteristics of the focal adhesion *in vitro* is the association of membrane proteins with the actin cytoskeleton, which facilitates force transduction. The *in vivo* counterpart of this adhesive association is not clear, although the epithelial adherens junction shares some features with the focal adhesion (Geiger et al., 1990) and is a site of tyrosine kinase enrichment and tyrosine phosphorylation (Tsukita et al., 1991). The adherens junction is a site of cell-to-cell adhesion found in the apico-lateral membranes of epithelial cells, but the transmembrane receptors associated with adherens junctions belong to the cadherin, rather than the integrin, family (Yap et al., 1997). Epithelial cells, therefore, have cell-to-cell adhesions in their apico-lateral regions and cell-to-ECM adhesions basally at the basement membrane. There is some evidence that FAK may be associated with either the apical or the basal regions of developing epithelia. Studies on human embryos showed a high expression of FAK in the apical region of epithelia in developing bronchi and kidney tubules (Tani et al., 1996). The same study also noted a different expression pattern in adult tissues, where FAK was localised primarily to the basal regions of the cells. Other studies in the developing mouse embryo have localised FAK to the basal region of the neural and somitic epithelia (Polte et al., 1994). It therefore appears that FAK is associated with apical regions of some developing epithelia and basal

regions of others, perhaps reflecting different developmental stages in epithelial morphogenesis. These studies suggest that FAK may not be directly associated with sites of cell-to-ECM adhesion in all cell types *in vivo*, although they do not show a specific localisation of FAK to an adhesive structure, either cell-to-cell or cell-to-ECM. In the absence of ultrastructural studies, there is currently no clear evidence that FAK is associated with adherens junctions *in vivo*.

In this connection, it is interesting to note that Pyk2 seems to be diffusely distributed throughout the cytoplasm of some cells (Zheng et al., 1998), or again associated with the apical cytoplasm of epithelia (Mitaka et al., 1997). There appears to be no consistent association of this molecule with cell-cell or cell-substratum points of adhesion.

SUMMARY

Embryonic morphogenesis is influenced by many factors, including the ECM. Integrins are a major family of cell-to-ECM receptors and their involvement in embryogenesis appears to be very broad, both spatially and temporally. FAK is the first member of a growing family of molecules that appear to transduce signals inside the cell in response to integrin adhesion. The regulation of FAK during development suggests it is important in the developmental process but it is not clear exactly what role it is playing. *In vitro* studies suggest an involvement in cell migration or cell death. The tensegrity theory provides an interesting model whereby cells respond to mechanical stimuli that alter the cytoskeleton. *In vitro*, FAK is activated in situations where contractile stress fibres are formed and so a situation may exist in the embryo where FAK is involved in signaling in response to generation of force, as in the tensegrity model. Interestingly, FAK is highly expressed in certain areas of the embryo that might be expected to encounter above average stresses such as the developing vasculature and the myotendinous junction. An important aspect of FAK signaling in development might be its response not only to integrin adhesion but also to growth factors. The fact that both growth factors and ECM are known to influence morphogenesis puts FAK in a position to regulate the cross-talk between the two types of receptor and integrate the signals into a single response.

AIMS OF THE THESIS

The work presented here is aimed at examining signaling proteins that are activated by cell adhesion to the ECM and their involvement in the early development of the chick embryo. The development of the chick embryo through the stages of gastrulation, which are the stages studied here, provides an excellent model for studying cell adhesion and migration events. The gastrulating chick embryo is a relatively simple and well-studied model that consists of different populations of cells interacting with the ECM and showing migratory and invasive behavior. The working hypothesis in this thesis was that adhesion to the ECM influences the behavior of cells during development, and may affect cell behavior and/or guidance of cell migration in the embryo. This work was directed at answering the following questions: whether cell adhesion-related signaling molecules, namely FAK, Pyk2 and paxillin, are involved in early stages of development; how the expression and activation of these molecules are influenced by changes in the ECM; and what functional role they have in embryonic cells. Answering these questions will give a better understanding of the role that these signaling molecules have in regulating cell behavior during development.

Chapter 3

MATERIALS AND METHODS

ANTIBODIES

Mouse monoclonal anti-focal adhesion kinase, mouse monoclonal anti-Pyk2, mouse monoclonal anti-phosphotyrosine (PY20), recombinant anti-phosphotyrosine (RC20) and mouse monoclonal anti-paxillin, were all purchased from Transduction Laboratories Inc. Rabbit polyclonal anti-chicken focal adhesion kinase and rabbit polyclonal anti-phosphotyrosine were obtained from Upstate Biotechnology Inc. Rabbit polyclonal anti-focal adhesion kinase (C-20) was purchased from Santa Cruz Biotechnology Inc. Mouse monoclonal anti-focal adhesion kinase (2A7) was a generous gift of Dr. J. Thomas Parsons. Mouse monoclonal anti-phosphotyrosine (P-3300) was purchased from Sigma Chemical Company.

EMBRYO DISSECTION AND HOMOGENISATION

Epiblast, mesoderm and endoderm tissues were dissected in Tyrode's saline ($CaCl_2$, $MgCl_2.6H_2O$, KCl, $NaHCO_3$, NaCl, $NaH_2PO_4.H_2O$, Glucose, pH 7.4), without enzymatic digestion, from chick embryos at stage 5 of Hamburger and Hamilton (1951) using chemically sharpened tungsten needles. Tissue was either homogenised immediately by brief sonication in homogenisation buffer (1mM Na_3VO_4 in PBS; NaCl, Na_2HPO_4, KH_2PO_4, pH7.4, plus protease inhibitor cocktail: 500μM AEBSF, 500μM EDTA, 1μM E-64, 1μM Leupeptin, 1μg/ml Aprotinin (Calbiochem Inc.)), or homogenised after cell culture (see below for cell culture conditions). For cell cultures, cells were rinsed in warm Tyrode's saline to remove culture medium and non-adherent cells. Remaining adherent cells were scraped off the coverslip, using a fine tungsten needle, into homogenisation buffer and sonicated briefly.

CELL CULTURE

Epiblast, endoderm and mesoderm tissues from chick embryos at stage 5 of Hamburger and Hamilton (1951) were dissected out and cultured on 25mm glass coverslips.

All cultures except those to be treated with oligonucleotide were grown in medium 199 (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL) and 0.1mg/ml gentamycin, and incubated at 37°C with 5% CO_2. For oligonucleotide

treatments, cells were grown in Opti-MEM medium (Gibco/BRL) supplemented with 10% FBS and 0.1mg/ml gentamycin, and incubated at 37°C with 5% CO_2 for 24 hours before oligonucleotide treatment (see below). For experiments requiring serum starved cultures, cells were allowed to adhere to the coverslips in medium 199 with 10% FBS and 0.1mg/ml gentamycin for 24 hours before the growing medium was changed and the cells were cultured in medium 199/0.1mg/ml gentamycin with no FBS for a further 24 hours.

For immunocytochemistry, interference reflection microscopy and oligonucleotide experiments, cells were grown on 50μg/ml fibronectin-coated coverslips. For immunoblot analysis, cells were grown on coverslips coated with either 50μg/ml laminin (Collaborative Biomedical Research Inc.) or 50μg/ml fibronectin (Collaborative Biomedical Research Inc.) or 60μg/ml Matrigel (Collaborative Biomedical Research Inc.). Coverslips were sonicated in double distilled water and cleaned with lens paper (Fisher Scientific Inc.) before being sterilised by autoclaving at 175°C for 1 hour. Substrata were made up to the appropriate concentration in medium 199 plus 0.1mg/ml gentamycin and incubated on the coverslip for 1 hour at room temperature to allow coating. Coverslips were then washed twice with medium 199 plus 0.1mg/ml gentamycin, to remove excess substratum before use.

CELL CULTURE IMMUNOCYTOCHEMISTRY

Cultures were were briefly rinsed in warm Tyrode's saline and fixed in 4% paraformaldehyde with 0.5% Tween 20 for 15 minutes. Fixative was removed by washing three times in PBS for 10 minutes each. Antigenic sites were blocked with 3% BSA in PBS for 45 minutes. For immunocytochemical localisation of FAK, polyclonal anti-chicken FAK antibody (UBI Inc.) at a dilution of 1:250 (10mg/ml) in 3% BSA/PBS, or in rabbit IgG (10mg/ml) for controls, for 3 hours at room temperature. Coverslips were washed overnight (18 hours) in 3% BSA/PBS. FITC conjugated goat anti-rabbit IgG secondary antibody was used at a dilution of 1:50 in 3% BSA/PBS for 30 minutes at 37°C. Coverslips were finally rinsed in PBS and mounted with Vectashield mounting medium (Vector Laboratories Inc.). For immunocytochemical localisation of paxillin and Pyk2, anti-paxillin (Transduction Laboratories Inc.) or anti-Pyk2 (Transduction Laboratories Inc.) were incubated for 1 hour at room temperature at a dilution of 1:25 in 3%

BSA/PBS. Coverslips were washed in 3% BSA/PBS for 15 minutes before incubation with secondary antibody, FITC conjugated goat anti-mouse IgG, at a dilution of 1:50 in 3% BSA/PBS for 30 minutes at 37°C. Coverslips were then rinsed in PBS and mounted with Vectashield mounting medium (Vector Laboratories Inc.)

Visualisation of biotin labeled oligonucleotides was done by blocking with 3%BSA/PBS for 30 minutes at room temperature before addition of FITC conjugated streptavidin (Calbiochem), diluted 1:50 in 3% BSA/PBS for 30 minutes at 37°C. Cultures were then washed for 30 minutes in 3% BSA/PBS before being mounted with Vectashield mounting medium (Vector Laboratories Inc.)

INTERFERENCE REFLECTION MICROSCOPY

Cultures grown on 25mm glass coverslips, for one-, three- or five-day time periods, were rinsed with warm Tyrode's saline and fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature and then kept in PBS. Fixed or unfixed cultures were viewed using a Leitz Diavert inverted microscope equipped with interference reflection optics.

WHOLE EMBRYO IMMUNOCYTOCHEMISTRY

Embryos at stage 5 of Hamburger and Hamilton (1951) were removed from their yolk and dissected in Tyrode's saline. Embryos were fixed in 4% paraformaldehyde for 4 hours after which the area pellucida was dissected out of the surrounding area opaca. Area pellucidas were washed in phosphate buffered saline (PBS) for 1 hour after fixation and permeabilised with 0.3% hydrogen peroxide in methanol for 15 minutes, followed by a further 15 minute PBS wash. Prior to antibody incubations antigenic sites were blocked in 1.5% BSA/PBS for 30 minutes at room temperature. Primary antibody, monoclonal anti-chicken FAK (Transduction Laboratories Inc.), was added at a dilution of 1:150 (16.5mg/ml) in 1.5% BSA/PBS for 18 hours at 4°C followed by 3 washes for 30 minutes each in PBS. Mouse IgG (16.5mg/ml) was used instead of anti-FAK in controls. Horseradish peroxidase-conjugated, goat anti-mouse IgG was added, at a dilution of 1:100 in PBS, for 1 hour at 4°C followed by 3 washes in PBS for 10 minutes each. Peroxidase was visualised using a mixture of 0.06% 3,3-diaminobenzidine (Sigma Inc.),

0.06% nickel ammonium sulphate, and 0.4% hydrogen peroxide in 0.1M Tris buffer. Washing the embryos in PBS for 15 minutes terminated the reaction. Stained embryos were dehydrated by a series of 15 minute washes in 50%, 70%, 90% and 100% alcohol before being cleared by a 30 minute wash in xylene. Embryos were then placed in molten paraplast embedding wax (Oxford labware Inc.) for four hours. The embedding wax was changed and the embryos left over night for the wax to infiltrate the tissue. Embryos were placed in moulds and the wax allowed to solidify. Wax embedded embryos were serial sectioning on a microtome at a thickness of 8μm. Sections were placed on slides and the wax removed by a 5 minute wash in Hemo-De (Fisher scientific Inc.) followed by rehydration of the sections by 15 minute washes in 100%, 70% and 50% alcohol and finally PBS. Sections were mounted in permount (Fisher scientific Inc.) and covered with a coverslip.

WHOLE EMBRYO ULTRASTRUCTURAL IMMUNOCYTOCHEMISTRY[2]

Embryos at stage 5 of Hamburger and Hamilton (1951) were dissected from their yolk and fixed in 2% paraformaldehyde/0.2% gluteraldehyde in PBS for 1 hour at 4°C. Embryos were then washed twice in PBS for 5 minutes and quenched in 50mM glycine in PBS for 15 minutes followed by 3 more 5 minute washes in PBS. Embryos were permeabilised with 0.5% Tween 20 in absolute methanol for 1 hour at 4°C. Antigenic sites were blocked by incubating the embryos in 1% BSA/0.5% Tween 20 in PBS (BSA buffer) for 1 hour at 4°C. Embryos were incubated with a 1:50 dilution of primary antibody, monoclonal anti-FAK (Transduction Laboratories Inc.), in BSA buffer for 16 hours. Control embryos were incubated with PBS in place of the primary antibody. Embryos were washed briefly in BSA buffer before being incubated with nanogold reagent (Nanoprobes Inc.) diluted 1:40 in BSA buffer, supplemented with 1% goat serum, for 16 hours at 4°C. Embryos were rinsed 3 times in PBS for 5 minutes followed by post fixation in 1% gluteraldehyde in PBS for 10 minutes at room temperature. Gluteraldehyde was removed by several 5 minute washes in deionised water and the embryos were then treated with HQ silver staining before further washes in deionised water. Treated embryos were

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[2] *Ultrastructural immunocytochemistry done by Sita Prasad and Dr. E. J. Sanders.*

dehydrated in a series of graded alcohol washes before being embedded in LR gold (Polysciences Inc.) and sectioned for visualisation under the electron microscope.

PROTEIN CONCENTRATION ASSAY

Protein concentrations of the homogenates were determined using a Bradford-based protein assay system (Biorad Inc.) done in 96 well plates and using BSA as a standard. Each sample was loaded in duplicate and the results averaged to give the protein concentration for that sample. Plates were read on a Titertek multiskan MC 96-well plate reader.

ELECTROPHORESIS

SDS-polyacrylamide gels were run by the method of Laemmli on a Mini-PROTEAN II electrophoresis system (Biorad Inc.). Equal amounts of protein (5-15μg as determined by protein concentration assay) from each tissue sample, or cell culture, were electrophoresed on 8% or 10% gels. 10μl of kaleidoscope broad range molecular weight standards were run in one lane of each gel. Gels were run at 200V, using a PowerPac 200 power supply (Biorad Inc.), until the dye front had reached the bottom of the gel. Proteins were transferred from the gels onto a supported nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Biorad). Transfer was done at 100V for 2 hours. Membranes were washed briefly with Ponceau red dye (Sigma Inc.) to visualise total protein. Ponceau red staining was removed during the blocking stage of the immunoblotting process.

IMMUNOBLOTTING

For immunoblots using monoclonal anti-FAK (Transduction Laboratories Inc., 1:1000), polyclonal anti-FAK (Santa Cruz Inc., 1:600), monoclonal anti-paxillin (Transduction laboratories Inc., 1:1000) or monoclonal anti-Pyk2 (Transduction Laboratories Inc., 1:1000) all membrane blocking, antibody incubation and washing steps were carried out in 3% skimmed milk in TTBS buffer (150mM Tris, 50mM NaCl, 0.1% Tween 20). In immunoblots for phosphotyrosine, carried out with the monoclonal anti-phosphotyrosine antibody, PY-20 (Transduction Laboratories Inc., 1:1500), RC20 (Transduction Laboratories Inc., 1:1000), or P-3300 (Sigma, 1:1000), all blocking,

antibody incubation and washing steps were carried out in 1.5% BSA-TTBS. Primary antibodies were incubated for 18 hours at 4°C followed by 3 washes for 30 minutes each, after which HRP-conjugated goat anti-mouse IgG (Transduction Laboratories Inc.) secondary antibody for monoclonal primary antibodies, or goat anti-rabbit IgG (Vector Laboratories Inc.) secondary antibody for polyclonal primary antibodies, was used at a dilution of 1:3000 for 2 hours at room temperature followed by three 30 minute washes. Immunoblots were visualised by exposure to Hyperfilm-ECL (Amersham Ltd.) after addition of enhanced chemiluminescent reagent (ECL, Amersham Ltd.). Molecular weights of visualised bands were determined by comparison to broad range molecular weight standards (Biorad Inc.).

Immunoblots were scanned to computer with a Microtek Scanmaker X6 and quantified by densitometric analysis on SigmaGel 1.0 software (Jandel Scientific Inc.) The intensities of each pixel in the band were measured and integrated to give a total value for band density. Relative levels of phosphorylation of FAK were calculated by taking the mean phosphotyrosine level, obtained by averaging quantified immunoblots, and dividing it by the mean FAK protein expression level for the same sample, also obtained by averaging quantified immunoblots.

To determine if the relationship between bands of different density on the immunoblots was linear, and directly proportional to the amount of protein present, whole chick embryo homogenate was run on an 8% gel at various known protein concentrations. These samples were blotted using anti-FAK or anti-paxillin monoclonal antibodies, as above. The band densities were analyzed by densitometry and plotted against the protein concentrations. The relationship between protein concentration and band density was found to be linear over the concentration range used in the experiments.

IMMUNOBLOT STRIPPING

Nitrocellulose membranes were stripped of antibodies by 45 minute incubation in stripping buffer (62.5mM Tris, 2%SDS, 100mM β-mercaptoethanol) at 55°C. Membranes were washed 3 times for 30 minutes each in TTBS prior to reblocking of the membrane and reprobing with different primary antibodies.

IMMUNOPRECIPITATION

For immunoprecipitation, embryos at stage 5 of Hamburger and Hamilton (1951) were dissected out and lysed in 15mM Tris-HCl and 1% SDS, with brief sonication. The lysate was diluted 10x with immunoprecipitation buffer (IPB; 10mM Tris-HCl, 1%NP-40, 5mM EDTA, 150mM NaCl, 0.5% deoxycholate, pH 7.5) to reduce the SDS concentration to 0.1%. One microgram of polyclonal anti-FAK antibody (Santa Cruz) and 50µl of a 25% slurry of protein-A coated sepharose beads (Sigma) in IPB were added to 300µl of lysate and incubated on a rotator for 16 hours at 4°C. The lysate was then centrifuged for 5 minutes at 15000 r.p.m. in an Eppendorf bench top centrifuge and the supernatant was removed from the protein-A beads. The beads were washed three times in immunoprecipitation buffer before being heated to 70°C for 5 minutes in 30 µl of 2x sample buffer (120mM Tris, 4%SDS, 20% glycerol). The sample buffer was then drawn off the sepharose beads and run in a single lane of an SDS-polyacrylamide gel, as above.

Control immunoprecipitations were done under the same conditions using a non-specific polyclonal rabbit IgG in place of the anti-FAK antibody or by replacing the lysate with PBS.

CO-IMMUNOPRECIPITATION

Tissue samples were solubilised in various co-immunoprecipitation buffers. Buffer 1 (150mM NaCl, 20mM Tris, 1% Nonidet P-40, 1mM Na_3VO_4, pH 7.6, plus protease inhibitor cocktail: 500µM AEBSF, 500µM EDTA, 1µM E-64, 1µM Leupeptin, 1µg/ml Aprotinin (Calbiochem Inc.)). Buffer 2 (150mM NaCl, 20mM Tris, 1% Tween 20, 1mM Na_3VO_4, pH 7.6, plus protease inhibitor cocktail: 500µM AEBSF, 500µM EDTA, 1µM E-64, 1µM Leupeptin, 1µg/ml Aprotinin (Calbiochem Inc.)). Buffer 3 (150mM NaCl, 20mM Tris, 1% Triton X-100, 1mM Na_3VO_4, pH 7.6, plus protease inhibitor cocktail: 500µM AEBSF, 500µM EDTA, 1µM E-64, 1µM Leupeptin, 1µg/ml Aprotinin (Calbiochem Inc.)). In each case tissue was solubilised at 4°C for 1 hour followed by gentle vortexing. The sample was centrifuged for 5 minutes at 15000 r.p.m. in an Eppendorf bench top centrifuge and the supernatant removed and used for co-immunoprecipitation. One microgram of polyclonal anti-FAK antibody (Santa Cruz) was added to the supernatant and incubated at 4°C for 30 minutes. 50µl of a 25% slurry of

protein-A coated sepharose beads (Sigma) in IPB was then added to the supernatant for 4 hours at 4°C on a rotator. The lysate was then centrifuged for 5 minutes at 15000 r.p.m. in an Eppendorf bench top centrifuge and the supernatant was removed from the protein-A beads. The beads were washed three times in immunoprecipitation buffer before being heated to 70°C for 5 minutes in 30 µl of 2x sample buffer (120mM Tris, 4%SDS, 20% glycerol) The sample buffer was then drawn off the sepharose beads and run in a single lane of an SDS-polyacrylamide gel, as above.

TUNEL

Cultured cells were examined for cell death using the TUNEL technique as follows. Cultured cells were grown on coated coverslips (as above), rinsed briefly in warm Tyrode's saline to remove excess culture medium and fixed in 0.5% Tween 20/4% paraformaldehyde in PBS for 15 minutes at room temperature. Cultures were then washed for 5 minutes in PBS before incubation in terminal transferase (TdT) buffer (30mM Tris, 140mM sodium cacodylate, 1mM cobalt chloride, pH 7.2) for 5 minutes at room temperature. The TUNEL reaction mixture (32.9µl deionised water, 2.6µl TdT buffer (Boehringer-Mannheim), 1.3µl cobalt chloride (Boehringer-Mannheim, 0.8µl Bio-16-dUTP (Boehringer-Mannheim), 2.4µl dUTP (Sigma), 0.8µl TdT (Boehringer-Mannheim)) was made up to a final volume of 40µl, allowing four cultures to be labeled with 10µl per culture. The cultures were incubated with the reaction mixture for 1 hour at 37°C and the reaction was terminated by washing the cultures in 2X saline sodium citrate buffer (0.3M sodium chloride, 30mM sodium citrate, pH 7) for 15 minutes at room temperature followed by a 5 minute PBS wash. The labeled cells were visualised by addition of streptavidin fluorescein at 1:50 in PBS for 30 minutes at 37°C. Cultures were then washed in PBS for 5 minutes and coverslips mounted onto slides using Vectashield mounting medium (Vector Laboratories Inc.). Cells undergoing apoptosis were identified with fluorescently labeled nuclei using this technique.

To label all nuclei within cell cultures diamidino-phenylindole (DAPI; Sigma Chemical Co.) was used at a concentration of 0.25µg/ml for 6 minutes, at room temperature in the dark. Cultures were rinsed in PBS and mounted using Vectashield mounting medium (Vector Laboratories Inc.).

ANTISENSE OLIGONUCLEOTIDES

Phosphorothioate oligonucleotides with a biotin label at the 5' end were made at the Core DNA lab, University of Calgary. One antisense oligonucleotide complementary to chicken FAK mRNA (5'-CTTGGTTCAAGCTGGATTAT-3') was used to block FAK protein expression and, as a control, the corresponding sense sequence (5'-ATAATCCAGCTTGAACCAAG-3') was used. A basic local alignment search tool (BLAST) was used to determine specificity of the sequence for FAK. The next closest match to the antisense sequence was found to be in chick embryo kinase 9 with 65% sequence similarity. The lyophilised oligonucleotides were reconstituted in double distilled water filtered through a 0.22 μm filter to give a final stock solution of 50μM. Oligonucleotides were introduced into cells using the cationic lipid, cytofectin (Glen research Inc.). Cytofectin was diluted 1:500 in serum free Opti-MEM medium (Gibco/BRL). In polystyrene 96 well plates 7.5μl of the diluted cytofectin was mixed with 12μl of oligonucleotide and 1.5μl of serum free Opti-MEM. Use of polypropylene was avoided to prevent lipid/oligonucleotide vesicles from sticking to the surface. Plates were then left at room temperature for 20 minutes. After 20 minutes, 9μl of Opti-MEM medium with 10% FBS was added to give a mixture with a final concentration of 20μM oligonucleotide, 3μg/ml cytofectin and 3%FBS. For experiments with 0% serum, 9μl of Opti-MEM without FBS was added. Medium was removed from cultures and replaced with the oligonucleotide/cytofectin medium using glass pipettes. Cultures were incubated for 24 hours at 37°C and 5% CO_2 with oligonucleotide/cytofectin medium before either fixation in 4% paraformaldehyde with 0.5% Tween 20 for 15 minutes at room temperature, for immunocytochemistry, or homogenisation in homogenisation buffer for immunoblotting. The efficacy of transfection was very high as judged by localisation of biotinylated oligonucleotides using FITC-conjugated streptavidin.

STATISTICAL ANALYSIS OF DATA

Data from the densitometric analysis of immunoblots on SigmaGel software was analyzed using paired t-test for comparison of two samples or with ANOVA with the Tukey post test for comparison of three or more samples together. Graphs show mean values with error bars showing standard error of mean.

For total nuclei counts with DAPI label, all labeled nuclei in every third field of view in the microscope were counted until the whole area of the culture was covered. The resulting counts were added together and tripled to give an estimated number of cells per culture. For TUNEL or DAPI labeled apoptotic nuclei, all positive cells in the culture were counted.

Cell areas and aspect ratios were measured after capturing the cell image on Image Pro software. Measurements were taken on ten different cells in each culture. The outline of the cell was traced and the area within the trace calculated using an arbitrary scale. The aspect ratio for each cell was calculated by measuring the long axis of the cell and dividing it by the measurement for the short axis. The closer the cell shape is to a circle, the closer the aspect ratio will be to one. More elongated cells will have an aspect ratio higher than one.

Chapter 4

EXPRESSION AND LOCALISATION OF ADHESION-RELATED SIGNALING MOLECULES

EXPRESSION OF FAK IN THE TISSUES OF THE GASTRULATING CHICK EMBRYO[3]

FAK localisation in cultured cells

FAK has previously been shown to localise to integrin-based focal adhesion complexes in cultured cells (Schaller et al., 1992). To examine the localisation of FAK in cultured chick embryo cells FAK visualisation using immunofluorescence was combined with interference reflection microscopy (IRM; Izzard and Lochner, 1980; Verschueren, 1985) a technique that allows direct visualisation of focal adhesions.

Cells from the epiblast, mesoderm and endoderm layers of gastrulating chick embryos were grown on fibronectin-coated glass coverslips and treated with antibodies against FAK for immunocytochemical localisation of FAK. In each of the three germ layers immunolabeling for FAK was localised to streak like regions on the ventral surface of the cell resembling focal adhesions (Figure 4a). Viewing the same cell under IRM showed that the labeling for FAK correlated with regions of the cell which appeared dark and were therefore in close contact with the substratum (Figure 4b). This confirmed the localisation of FAK to focal adhesions.

In the mesoderm cells, however, FAK also localised to broader regions of the lamellipodia (Figure 4c), that, when viewed under IRM (Figure 4d) were seen as grey patches. These grey patches may represent regions that have been termed "close contacts" (Izzard and Lochner, 1980). Close contacts have not been as well studied as focal adhesions, but they appear to be distinct adhesion sites associated with cell spreading, and are more common in highly motile cells. Control cultures showed no fluorescence in any regions of the cells (Figure 4e).

Another unusual feature was the strong staining for FAK in the nucleus, but not the nucleolus (Figure 4c). This nuclear staining increased during the first three days of culture, after which it persisted until the fifth day, which was the longest time period studied.

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[3] *Parts of this chapter have been published in, Ridyard, M. S. and Sanders, E. J. (1998) Cellular phenotypic transformation during early embryogenesis: a role for focal adhesion kinase? Biochem. Cell Biol. 76: 45-58*

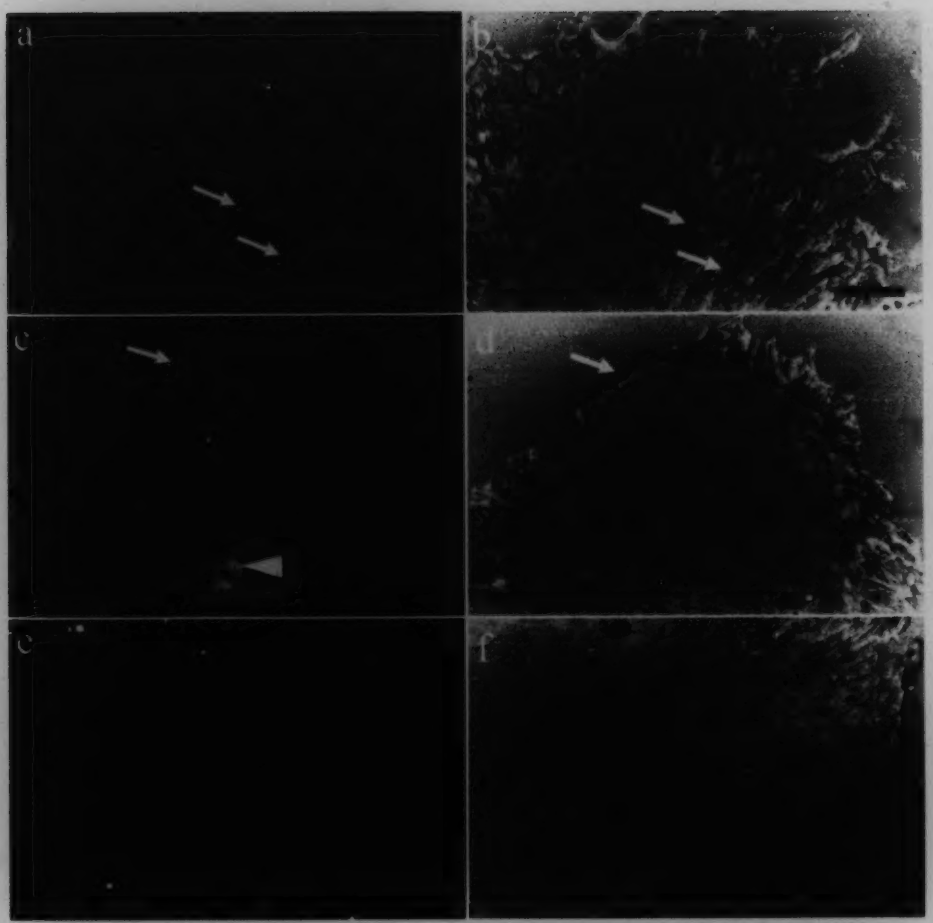


Figure 4. Cultured chick embryo cells immunolabeled for FAK. (a) FAK is localised to focal adhesions in endoderm cells (arrow). (b) The same cell as in (a), viewed by interference reflection microscopy to show focal adhesions. (c) FAK is localised to focal adhesions as well as to broad regions (arrow) and to the nucleus (arrowhead) in mesoderm cells. (d) The same cell as in (c) viewed by interference reflection microscopy for focal and close contacts. (e) Control where anti-FAK antibody was replaced with mouse IgG shows no labeling. (f) The same cell as in (e) viewed by interference reflection microscopy for focal adhesions. Magnification x500; Scale bar = 25μm.

FAK LOCALISATION IN SECTIONED EMBRYOS

To visualise the localisation of FAK in gastrulating chick embryos the embryos were immunohistochemicaly labeled using monoclonal antibody to FAK and transversely sectioned on a microtome.

FAK immunoreactivity appeared in a spatially restricted pattern in the stage 5 gastrulating embryo, being localised to specific regions within each germ layer (Figure 5). The primary site of immunoreactivity was the upper epithelial epiblast layer, with only limited regions of the mesoderm and endoderm layers showing any significant staining.

Sections taken through Hensen's node and the primitive streak near to Hensen's node showed that the immunolabeling for FAK in the epiblast appeared throughout the thickness of the epiblast but immunoreactivity in Hensen's node and the primitive streak itself, where the phenotypic transformation of the epiblast is occurring, was restricted to a narrow apical zone (Figure 5b,c).

In more caudal regions of the embryo FAK immunoreactivity was restricted to a narrow apical zone throughout the entire epiblast with no change in staining pattern between the primitive streak and non-primitive streak regions (Figure 5d).

Strong immunoreactivity appeared in the mesoderm in the region where the cells invade the ECM-filled space between the epiblast and the endoderm, ventro-lateral to the primitive streak (Figure 5c,d). In the region where the mesoderm migrates away from the primitive streak, FAK immunolabeling was reduced so that the peripheral mesoderm showed very little FAK immunoreactivity (Figure 5c,d). The lower endoderm layer showed the same staining pattern as the mesoderm layer, with strong immunolabeling at the primitive streak and less in the more peripheral areas (Figure 5c,d).

Close examination of the primitive streak clearly shows the lack of FAK immunoreactivity in this structure apart from the narrow apical region of the epiblast (Figure 6a arrow) and the mesoderm and endoderm exiting the primitive streak (Figure 6b arrow).

Control embryos, incubated with non-specific IgG in place of antibodies to chicken FAK, showed no labeling (Figures 5e and 6c).

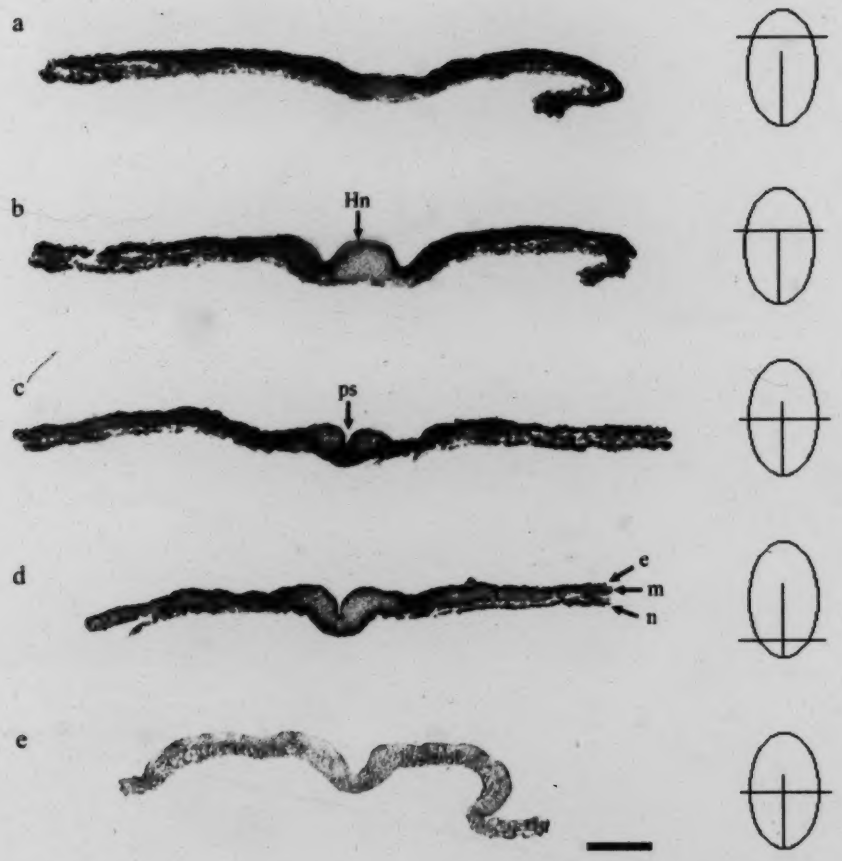


Figure 5. Sections through a stage five chick embryo immunolabeled for FAK. (a-d) The sections are at progressively more caudal levels as indicated by the diagrams at the right. Labeling is heavy in the upper epiblast layer, except in the regions of Hensen's node and the primitive streak. At the more caudal levels, staining is primarily at the apical side of the epiblast layer. (e) Control section in which monoclonal anti-FAK antibody was replaced with mouse IgG. Hn-Hensen's node; ps-primitive streak; e-epiblast; m-mesoderm; n-endoderm. Magnification x50; Scale bar = 200μm.

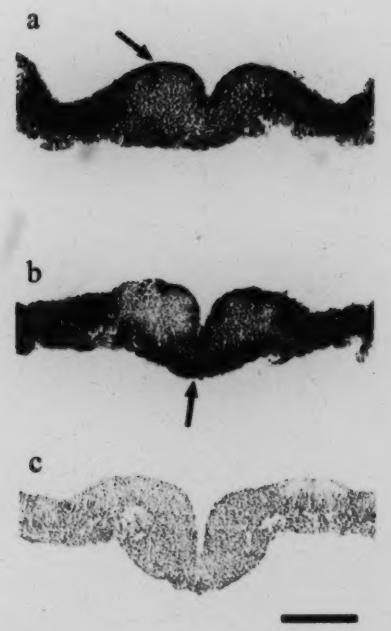


Figure 6. Sections through the primitive streak immunolabeled for FAK. (a) A transverse section through the primitive streak showing the apical cytoplasmic staining in this region (arrow). (b) A similar section to that in (a), showing immunoreactivity in the mesoderm and endoderm cells as they emerge from the primitive streak (arrow). (c) Control section in which the primary antibody was replaced with mouse IgG. Magnification x250; Scale bar = 100μm.

Ultrastructural localisation of FAK

To examine the subcellular localisation of FAK *in vitro* and *in vivo* cultured cells and whole embryos were immunolabeled with anti-FAK antibodies and visualised using nanogold reagent. Cultures and embryos were sectioned and viewed under transmission electron microscope.

In cultured mesoderm cells localisation of anti-FAK antibody was observed to be similar in pattern to the results seen using light microscopy. Sections through the advancing lamelleipodium showed FAK immunoreactivity in small clusters on the ventral surface of the cell (Figure 7a). In sections through the nucleus immunolabeling was observed in a dispersed pattern throughout the nucleus (Figure 7b).

Sections through whole embryos showed FAK immunoreactivity in the apical cytoplasm of epiblast cells. There was no defined localisation of FAK to specific structures within the apical region of the cell, rather, a diffuse distribution of FAK immunolabeling was seen. (Figure 7c). It was notable that FAK did not show localisation to any region of cell-to-cell adhesion including adherens junctions that were clearly visible in the apico-lateral membranes in some sections (Figure 7c, arrowhead).

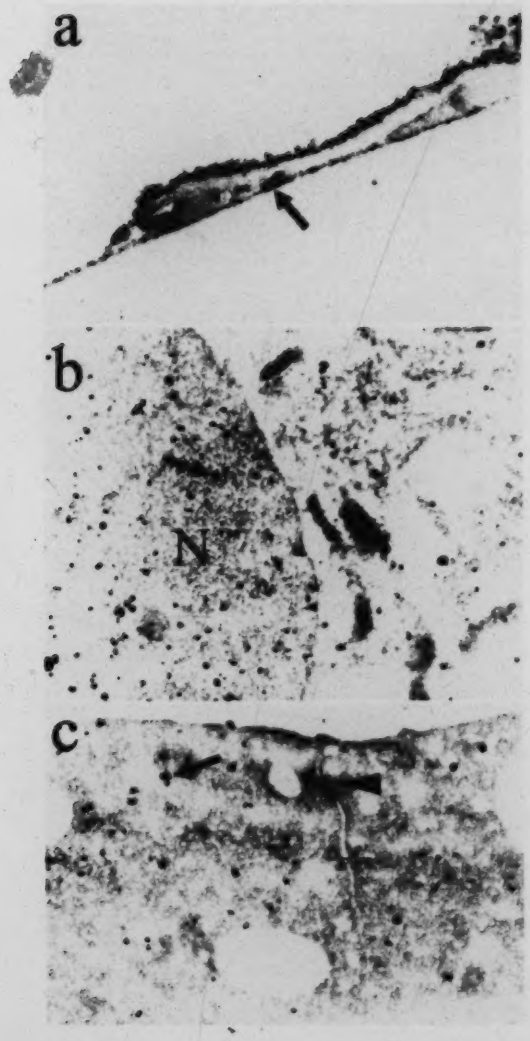


Figure 7. Ultrastructural localisation of FAK in chick embryo cells. (a) A transverse section through a mesoderm cell grown in culture showing localisation of anti-FAK antibodies to sites on the ventral plasma membrane (arrow). Magnification x50200(b) A transverse section through a mesoderm cell grown in culture showing localisation of anti-FAK antibodies (arrow) to the nucleus (N). Magnification x18150 (c) A transverse section through an epiblast cell of a gastrulating chick embryo. Anti-FAK antibodies do not localise to specific structures within the cell but are concentrated in the apical cytoplasm (arrow). FAK does not localise to adherens junctions (arrowhead). Magnification x20600.

Characterisation of antibodies on immunoblots

Specificity of the anti-FAK antibody, monoclonal 77, used in the previous experiments was determined by immunoblotting samples of whole embryo homogenate with monoclonal 77 antibody (Figure 8a, lane 2) and comparing it with immunoblots using monoclonal antibody 2A7, a commonly used anti-FAK antibody (Figure 8a, lane 1). The immunoblot with monoclonal 77 showed a single band at approximately 125kDa. The immunoblot with monoclonal 2A7 showed several bands with a major band at approximately 125kDa. Monoclonal 77 appears to be very specific and recognises a protein at the correct molecular weight for FAK.

To determine if monoclonal 77 was recognising a specific isoform of FAK or the total cellular FAK it was used to immunoblot whole embryo samples (Figure 8b lane 1) and compared to a polyclonal anti-FAK, antibody C-20 (Figure 8b lane 2). The polyclonal antibody may pick out different isoforms of FAK resulting in greater overall detection of FAK. Quantification of the 125kDa bands on the immunoblots, by densitometry, showed that polyclonal C-20 gave a slightly higher average band density (Figure 8c), although it was not significantly different from monoclonal 77 when repeated immunoblots were quantified and analysed by paired t-test ($p=0.0972$).

In all the following experiments monoclonal anti-FAK refers to the monoclonal 77 antibody and polyclonal anti-FAK refers to the polyclonal C-20 antibody. Monoclonal anti-paxillin refers to the monoclonal 349 antibody.

To determine if there was a linear relationship between band density on immunoblots and protein concentration, known dilutions of whole embryo homogenate were loaded onto polyacrylamide gels and immunoblotted with monoclonal anti-FAK (Figure 9a) or monoclonal anti-paxillin (Figure 9c) antibodies. The resulting bands were analysed by densitometry and the relationship between band density and the amount of protein loaded in each lane was plotted for each immunoblot (Figure 9b,d). The relationship was found to be linear for both antibodies, suggesting the level of protein expression in a sample can be determined directly by quantifying the band density of each protein on an immunoblot.

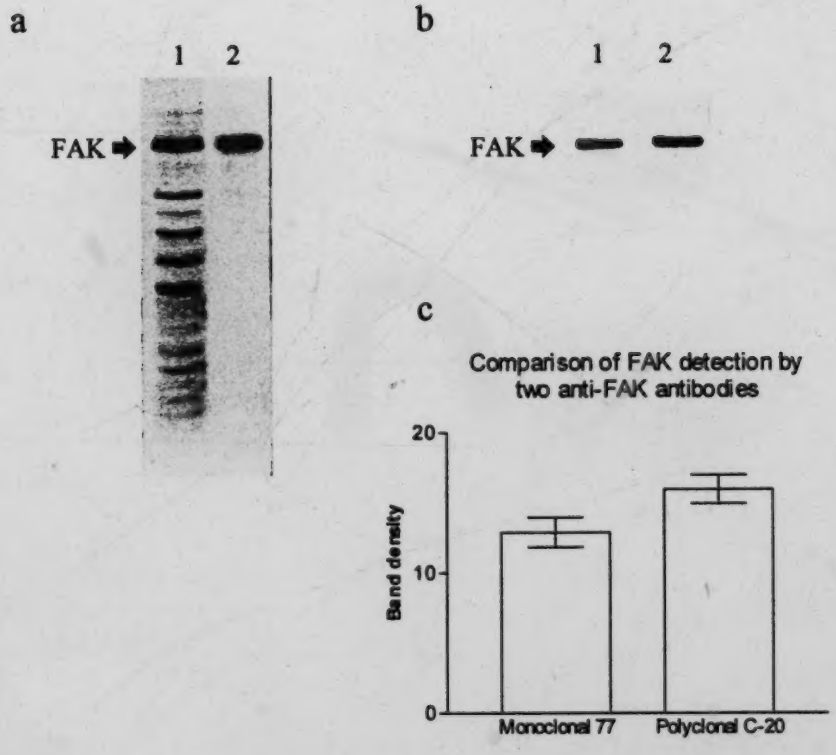


Figure 8. Immunoblot comparison of different anti-FAK antibodies. (a) Immunoblot of whole embryo samples using monoclonal 2A7 (lane 1) or monoclonal 77, anti-FAK (lane 2). (b) Immunoblot showing the detection of FAK by monoclonal 77 (lane 1) and polyclonal C-20 (lane 2). (c) Quantification of 125kDa bands on monoclonal 77 and polyclonal C-20 blots, obtained by densitometry (n=4).

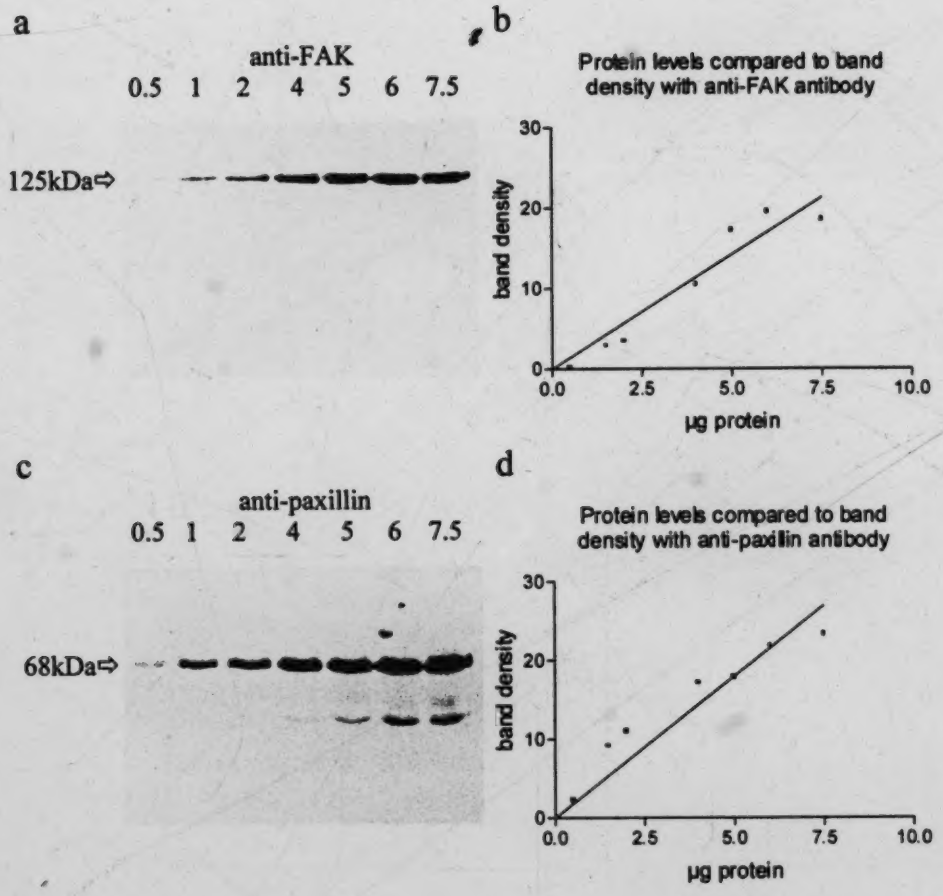


Figure 9. Relationship between protein level and band density on immunoblots. (a) Immunoblot using monoclonal anti-FAK. Lanes are marked with protein loaded in µg. (b) Plot of the band densities in (a) verses the amount of protein loaded in each lane. (c) Immunoblot after stripping the membrane in (a) of antibodies and reprobing with monoclonal anti-paxillin. (d) Plot of the band densities in (d) verses the amount of protein loaded in each lane.

Immunoblot analysis of FAK in embryonic tissues

Immunoblots were carried out on samples of individual germ layers in order to quantify the levels of FAK protein expressed *in vivo*, and to examine the levels of tyrosine phosphorylation, which is an indicator of kinase activity. Immunoblots using monoclonal anti-FAK showed two major bands for each tissue at molecular weights of 125kDa and 105kDa (Figure 10a). The 125kDa band represents the full-length chicken FAK protein. The band appearing at 105kDa may represent a proteolytic fragment of FAK.

Densitometric analysis of the 125kDa band on FAK immunoblots showed a consistent trend of high levels of expression of FAK in the epiblast, with the mesoderm and endoderm showing a reduction in FAK expression when compared to the epiblast although there was no significant difference between the levels of FAK when analysed by ANOVA (Figure 10c). However, this pattern correlated well with the FAK immunoreactivity seen in the whole embryo sections, which showed only limited staining associated with mesoderm and endoderm cells in the region of the primitive streak (Figures 5 and 6).

The nitrocellulose membranes were stripped of FAK antibodies and reprobed with anti-phosphotyrosine antibodies (clone PY-20) to examine levels of FAK tyrosine phosphorylation (Figure 10b). Bands on the phosphotyrosine immunoblots, at a molecular weight 125kDa, corresponding to the band at 125kDa on the FAK immunoblots, were analyzed by densitometry (Figure 10d). Results showed differing levels of phosphotyrosine between the germ layers.

When FAK phosphotyrosine was expressed in relation to the amount of FAK protein, it became clear that there was no significant difference between the phosphotyrosine levels of FAK in each germ layer (Figure 10e).

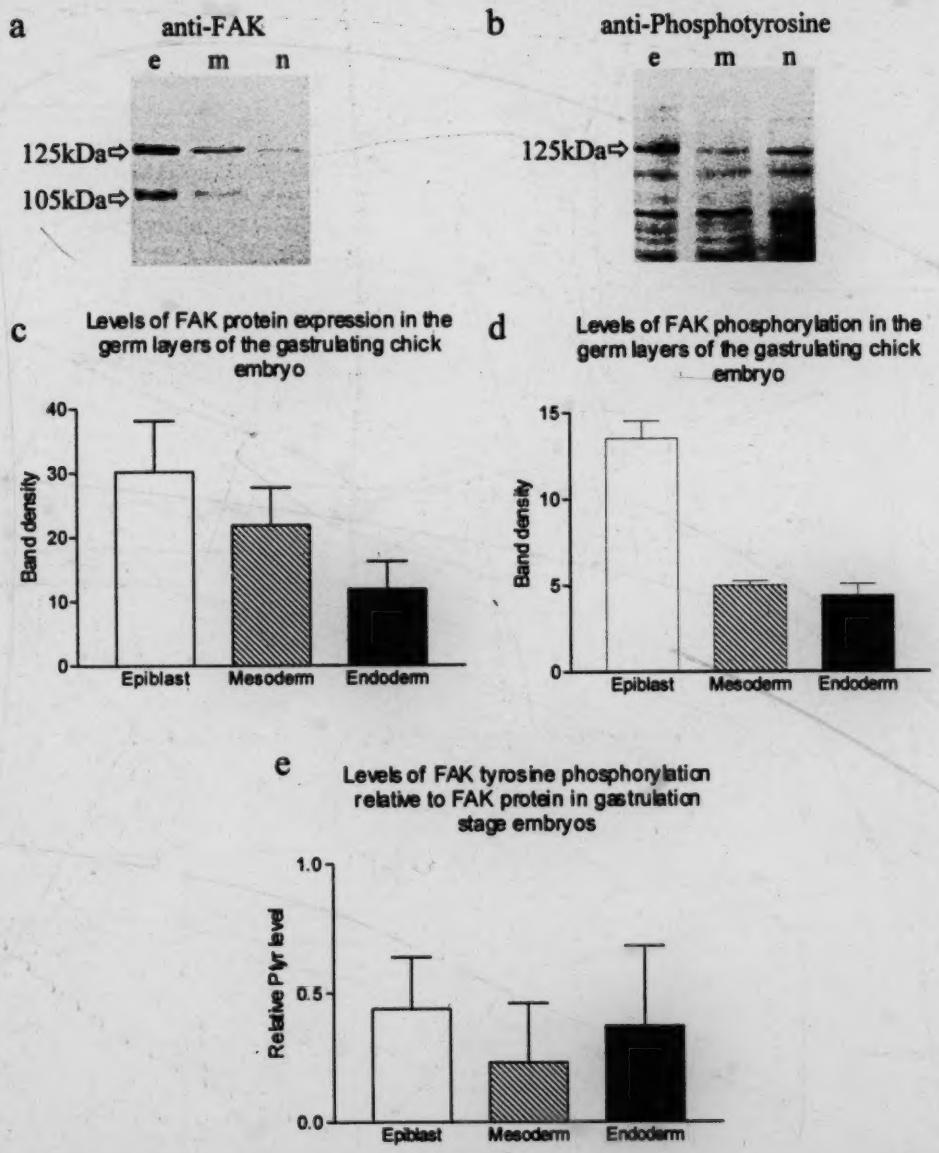
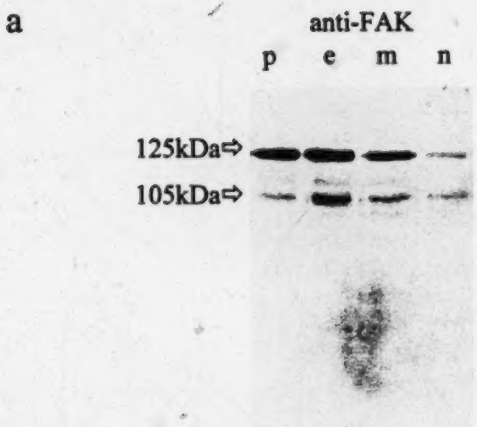


Figure 10. FAK expression and phosphorylation in the tissues from the stage 5 chick embryo. (a) Epiblast (lane e), mesoderm (lane m) and endoderm (lane n) blotted with monoclonal anti-FAK antibody (b) The same lanes shown in (a) stripped and re-blotted with antibody to phosphotyrosine. (c) Average level of FAK expression as determined by densitometry of immunoblots (n=6). (d) Average level of FAK phosphotyrosine as determined by densitometry of immunoblots (n=2). (e) The levels in (d) divided by the levels in (c), to provide levels of phosphorylation relative to levels of FAK expression. Vertical bars on all histograms indicate the standard error of the mean.

Immunoblot analysis of FAK in the primitive streak

The apparent change in the expression level of FAK at the primitive streak, indicated by the immunohistochemical results, was further studied by dissection and immunoblotting of the primitive streak tissue itself. The three germ layers were separated and the primitive streak removed from the embryo. Equal levels of protein were loaded onto an SDS-polyacrylamide gel and the tissues were examined by immunoblotting, as above, using monoclonal anti-FAK antibodies (Figure 11a). The results showed that the level of expression of FAK in the primitive streak region was between that of the epiblast and mesoderm. Densitometric analysis of the FAK bands in each sample showed that the primitive streak region expressed 80% the level of FAK protein of the epiblast, compared to the mesoderm which showed 60% the level of FAK protein of the epiblast (Figure 11b).



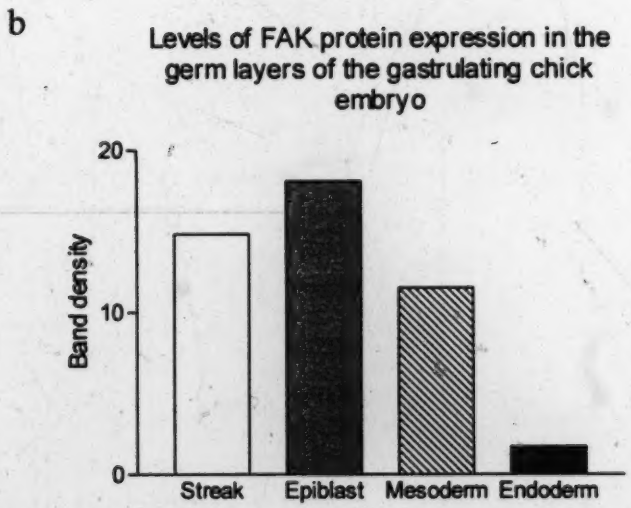


Figure 11. FAK expression in the tissues from the stage 5 chick embryo including the primitive streak. (a) Primitive streak (lane p), epiblast (lane e), mesoderm (lane m) and endoderm (lane n) blotted with monoclonal antibodies to FAK. (b) Level of FAK expression as determined by densitometry of immunoblots, (n=1).

Immunoprecipitation of FAK

To determine if the band at 125kDa on phosphotyrosine immunoblots specifically represented phosphorylated FAK, FAK was immunoprecipitated from the supernatant of whole embryo homogenate and the immunoprecipitate and supernatant samples were analysed by immunoblot.

To avoid non-specific bands from appearing on the immunoblot resulting from cross reactivity between the secondary antibody used in the immunoblot (horseradish peroxidase conjugated goat anti-mouse antibody) with the heavy and light chains of the antibody used in the immunoprecipitation, the rabbit polyclonal anti-FAK antibody was used for immunoprecipitation and the mouse monoclonal anti-FAK antibody was used for immunoblots.

Immunoblots showed that FAK was specifically immunoprecipitated (Figure 12a, lane 1). Comparison of the supernatant before (Figure 12a, lane 3) and after (Figure 12a, lane 2) immunoprecipitation showed that FAK was removed from the supernatant by the immunoprecipitation. Stripping and reprobing the same membrane with PY-20 anti-phosphotyrosine antibodies showed that the immunoprecipitated FAK was highly phosphorylated (Figure 12b lane 1). The corresponding band at 125kDa was reduced after immunoprecipitation of FAK (Figure 12b, lane 2) in comparison with the non-precipitated supernatant (Figure 12b, lane 3), indicating that this phosphotyrosine band represented phosphorylated FAK.

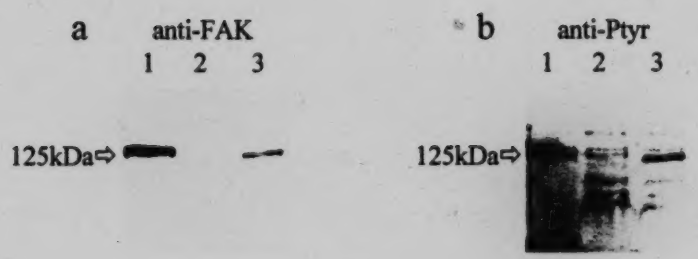


Figure 12. Immunoprecipitation of FAK from whole embryo lysate. (a) Immunoprecipitated FAK (lane 1), supernatant after immunoprecipitation (lane 2), supernatant before immunoprecipitation (lane 3). (b) The same lanes as shown in (a), stripped and reprobed with antibody to phosphotyrosine. Lane 2 shows that the level of phosphotyrosine at 125kDa is greatly reduced after immunoprecipitation of FAK.

Comparison of FAK expression *in vivo* and *in vitro*

To evaluate if the transition from *in vivo* conditions to two-dimensional *in vitro* culture conditions is associated with a change in the level of FAK protein expression, tissue from the three germ layers was dissected from gastrulation stage embryos and either homogenised immediately or grown on fibronectin-coated glass coverslips for 24 hours before homogenisation. Equal amounts of protein from each sample were run on an 8% SDS-polyacrylamide gel and the levels of FAK protein in each sample were examined by immunoblotting.

Immunoblot analysis using monoclonal anti-FAK showed a major band in each lane at 125kDa that represented the full length FAK protein (Figure 13a). This band was quantified by densitometry (Figure 13b).

Comparison of the levels of FAK protein expression for each germ layer showed that there were differing responses to *in vitro* culture conditions for each germ layer. The epiblast layer showed an average 35% decrease in the expression level of FAK protein in cells grown *in vitro* compared to those *in vivo* (Figure 13b). In contrast, mesoderm cells grown *in vitro* showed no change in the level of FAK protein expression compared to FAK protein levels *in vivo* (Figure 13b). Endoderm cells grown *in vitro* showed an increase of 60% in the level of FAK protein expression compared to FAK protein levels *in vivo* (Figure 13b). These differences indicated trends in protein levels the significance of which could not be determined due to the low number of experiments.

Epiblast and endoderm samples from *in vivo* conditions showed two extra bands at 115kDa and 105kDa. These were minor bands compared to the band at 125kDa in each lane.

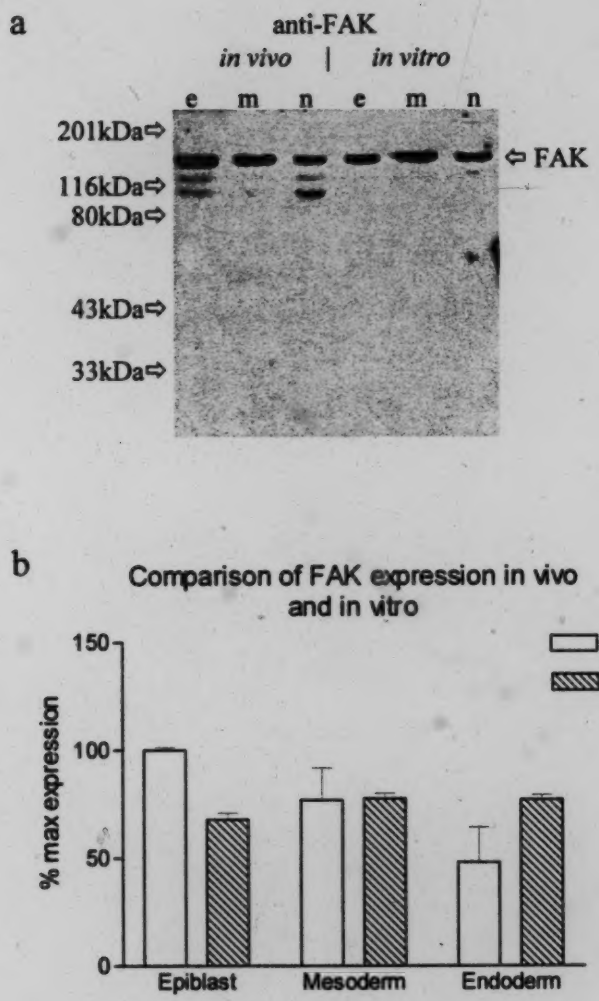


Figure 13. Comparison of FAK expression levels in the tissues of the stage 5 chick embryo and in cultures grown on fibronectin. (a) Immunoblot showing FAK protein levels in the epiblast (lanes e), mesoderm (lanes m) and endoderm (lanes n) from tissue dissected directly from each germ layer (*in vivo*) and from tissue cultured on fibronectin for 24 hours (*in vitro*). Molecular weight markers are indicated at the left. (b) Average level of FAK expression as determined by densitometry of immunoblots, (n=2). Bars represent the standard error from the mean.

EXPRESSION OF PYK2 IN THE TISSUES OF THE GASTRULATING CHICK EMBRYO

Pyk2 localisation in cultured cells

Cells from each of the three germ layers of the gastrulating chick embryo were grown on fibronectin-coated coverslips and examined for Pyk2 localisation. In contrast to the immunolabeling for FAK (Figure 4), Pyk2 immunolabeling showed no localisation to any sites of cell adhesion (Figure 14a,c,e). Epiblast, mesoderm and endoderm cultures, however, all showed some localisation of Pyk2 immunostaining in the nuclei.

The nuclear localisation was confirmed by double labeling of the cultures with DAPI, a DNA stain that allows visualisation of the nuclei (Figure 14b,d,f). The immunoreactivity for Pyk2 appeared to be in the nucleus but not the nucleolus. Also, the Pyk2 immunolabeling in the mesoderm nuclei (Figure 14c) appeared to be more intense than the staining in either the epiblast (Figure 14a) or endoderm (Figure 14e) nuclei. This is similar to the immunoreactivity observed for FAK in cultured cells where the mesoderm cultures appeared to have nuclear localisation of FAK (Figure 4c) that was not as evident in epiblast or endoderm cultures.

Close examination of the nuclear labeling for Pyk2 compared to the DAPI labeling revealed that not all of the nuclei were labeled for Pyk2 in each culture. All epiblast nuclei appeared to be labeled for Pyk2 although this was hard to determine as the epiblast cells were slow to spread and only a small region of cells around the edge of the culture were spread enough to make clear visualisation of the nuclei possible (Figure14a,b). The mesoderm cultures showed a small percentage of nuclei that were only visible with the DAPI stain and did not label for Pyk2 (Figure 14c,d). The endoderm also showed some DAPI labeled nuclei that did not appear to label for Pyk2 although there appeared to be more non-Pyk2 labeled nuclei in the endoderm cultures than in the mesoderm cultures (Figure 14e,f).

Cultures in which the antibody to Pyk2 was replaced with mouse IgG showed no immunoreactivity apart from the DAPI labeling of the nuclei (Figure 14g,h).

Figure 14. Cultured chick embryo cells immunolabeled for Pyk2 and DNA. (a,c,e,g) Immunolabeling for Pyk2. (b,d,f,h) DAPI labeling for nuclei. (a,b) Epiblast shows nuclear labeling for Pyk2. (c,d) Mesoderm shows strong nuclear labeling for Pyk2 although not all nuclei are labeled (arrows). (e,f) Endoderm shows nuclear labeling for Pyk2 although not all nuclei are labeled (arrows). (g,h) Control cultures where anti-Pyk2 was replaced with mouse IgG show no labeling. Magnification x300; Scale bar = 100μm.

Pyk2 localisation in sectioned embryos

To visualise the localisation of Pyk2 in gastrulating chick embryos the embryos were immunohistochemicaly labeled using monoclonal antibody to Pyk2 and transversely sectioned on a microtome.

The embryos that were treated with antibodies to Pyk2 (Figure 15a-d) showed no specific localisation of Pyk2 but did show some staining that appeared throughout the embryo and, when compared to control embryos that were treated with mouse IgG in place of the antibody to Pyk2 (Figure 15e), was stronger in the anterior (Figure 15a) than the posterior (Figure 15d) of the embryo.

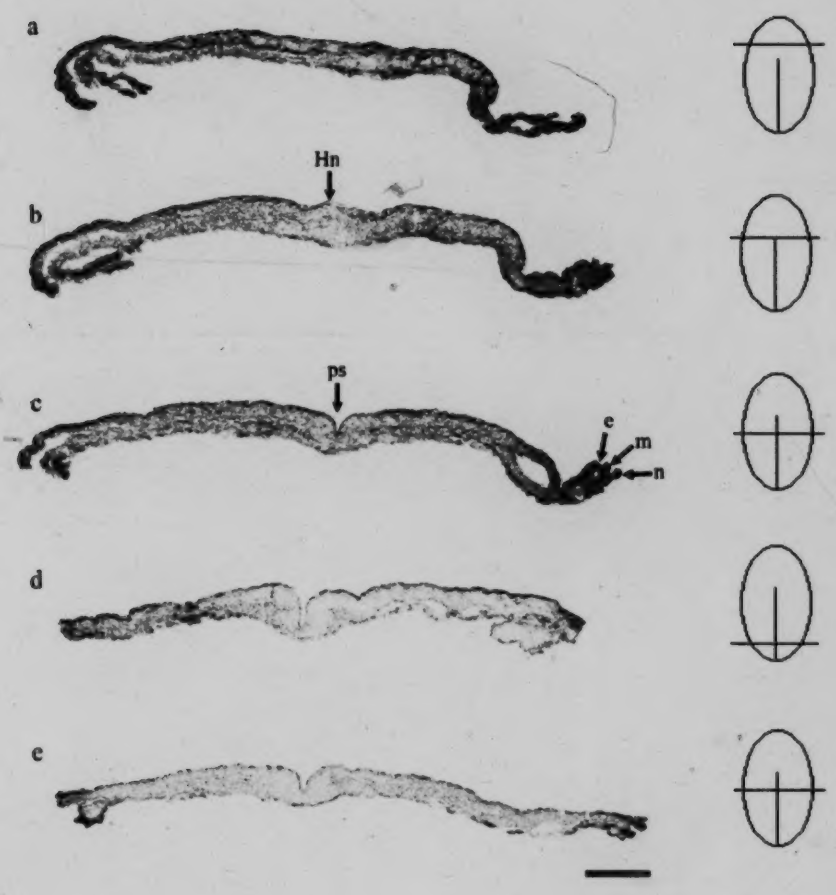


Figure 15. Sections through a stage five chick embryo immunolabeled for Pyk2. (a-d) Transverse sections are taken at different levels through the same embryo as indicated by the diagrams at the right. Immunolabeling for Pyk2 is stronger in the anterior section (a), than the posterior (d). (e) Transverse section through an embryo labeled with mouse IgG in place of anti-Pyk2. Hn-Hensen's node, ps-primitive streak, e-epiblast, m-mesoderm, n-endoderm. Magnification x50; Scale bar = 200μm.

Comparison of Pyk2 expression *in vivo* and *in vitro*

To evaluate if the transition from *in vivo* conditions to two-dimensional *in vitro* culture conditions is associated with a change in the level of Pyk2 protein expression, tissue from the three germ layers was dissected from gastrulation stage embryos and either homogenised immediately or grown on fibronectin-coated glass coverslips for 24 hours before homogenisation. Equal amounts of protein from each sample were run on an 8% SDS-polyacrylamide gel and the levels of Pyk2 protein in each sample were examined by immunoblotting.

Immunoblot analysis using monoclonal anti-Pyk2 showed a major band in each lane at 123kDa that represented the full length Pyk2 protein (Figure 16a). This band was quantified by densitometry (Figure 16b).

Comparison of the levels of Pyk2 protein expression for each germ layer showed that there were differing responses to *in vitro* culture conditions for each germ layer. There was an indication of overall increases in Pyk2 expression when each of the tissues were grown *in vitro*, with epiblast, mesoderm and endoderm cells showing an average increases of 250%, 325% and 430% respectively in the expression level of Pyk2 protein *in vitro* compared to *in vivo* levels (Figure 16b). These differences indicated trends in protein levels the significance of which could not be determined due to the low number of experiments.

All lanes on the immunoblot showed a second band at approximately 50kDa. This band showed comparable intensity to some of the bands at 123kDa and may represent a fragment of the Pyk2 protein.

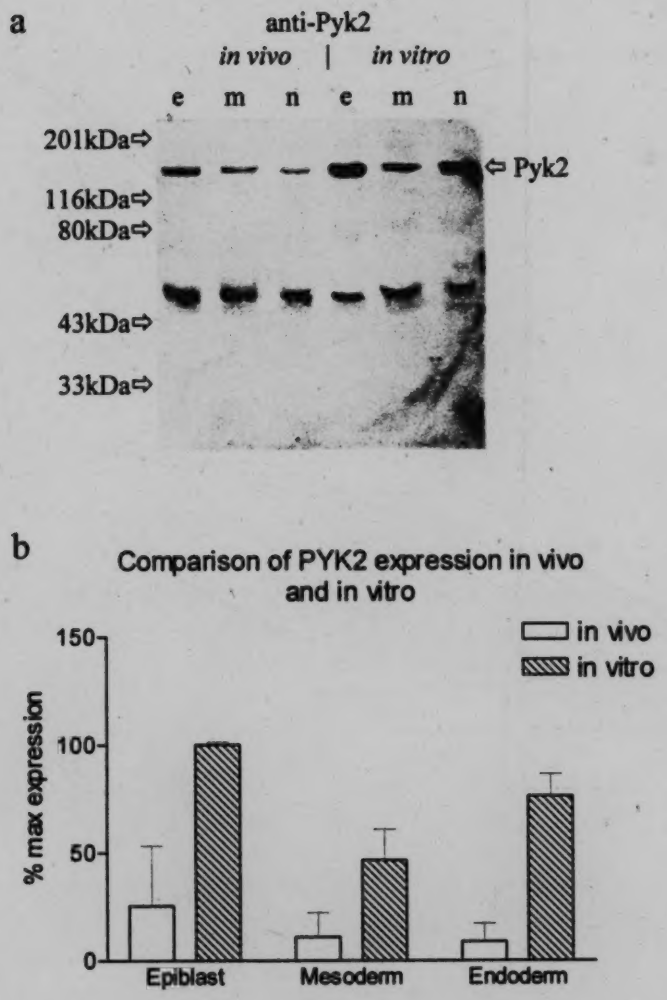


Figure 16. Comparison of Pyk2 expression levels in the tissues of the stage 5 chick embryo and in cultures grown on fibronectin. (a) Immunoblot showing Pyk2 protein levels in the epiblast (lanes e), mesoderm (lanes m) and endoderm (lanes n) from tissue dissected directly from each germ layer (in vivo) and from tissue cultured on fibronectin for 24 hours (in vitro). Molecular weight markers are indicated at the left (b) Average level of Pyk2 expression as determined by densitometry of immunoblots, (n=2). Bars represent the standard error from the mean.

EXPRESSION OF PAXILLIN IN THE TISSUES OF THE GASTRULATING CHICK EMBRYO

Paxillin localisation in cultured cells

Cells from each of the three germ layers of the gastrulating chick embryo were grown on fibronectin-coated glass coverslips and examined for paxillin localisation by immunolabeling with anti-paxillin antibodies.

Epiblast (Figure 17a), mesoderm (Figure 17b) and endoderm (Figure 17c) all show paxillin labeling in small streaks at the periphery of the cells that represents localisation in focal adhesions. The mesoderm cells also show paxillin localisation in broad areas, again at the periphery of the cells, that represents localisation in close contacts (Figure 17b, arrowhead).

Figure 17. Cultured chick embryo cells immunolabeled for paxillin. (a) Epiblast cells show paxillin immunoreactivity in focal adhesions (arrow). (b) Mesoderm cells show paxillin immunoreactivity in focal adhesions (arrow) and also in close contacts (arrowhead). (c) Endoderm cells show paxillin immunoreactivity in focal adhesions (arrow). (d) Control cultures where anti-paxillin was replaced with mouse IgG show no labeling. Magnification x500; Scale bar = 25μm.

Paxillin localisation in sectioned embryos

To visualise the localisation of paxillin in gastrulating chick embryos the embryos were immunohistochemicaly labeled using monoclonal antibody to paxillin and transversely sectioned on a microtome.

Immunoreactivity for paxillin appeared in specific regions of the embryo sections. The epiblast layer showed specific immunolabeling in what appeared to be small groups of cells, mainly in the apical layer of the epiblast (Figure 18b and 19b). Regions of the mesoderm and endoderm showed immunolabeling although the immunoreactivity was not uniform throughout these layers, with sections from some regions of the embryo showing stronger labeling than others. (Figure 18a,c and 19a). This pattern of immunoreactivity was seen in seven different embryos immunolabeled for paxillin.

Control embryos, incubated with non-specific mouse IgG in place of antibodies to paxillin, showed no labeling (Figure 18d and 19c)

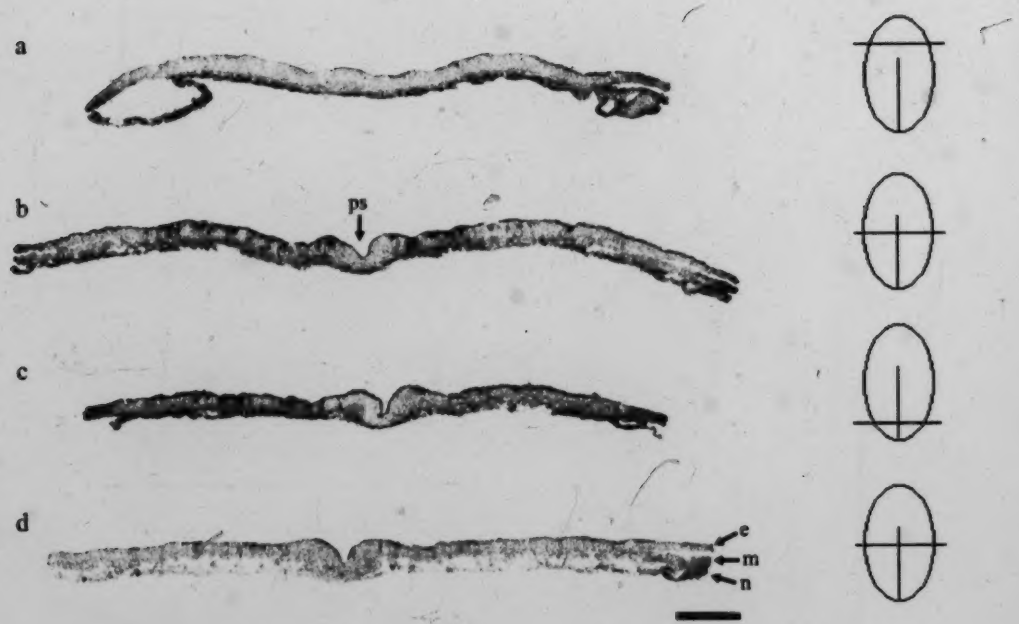


Figure 18. Sections through a stage five chick embryo immunolabeled for paxillin. (a-c) Transverse sections are taken at different levels through the same embryo as indicated by the diagrams at the right. (d) Transverse section through an embryo stained with mouse IgG in place of anti-paxillin. ps-primitive streak, e-epiblast, m-mesoderm, n-endoderm. Magnification x50; Scale bar = 200μm.

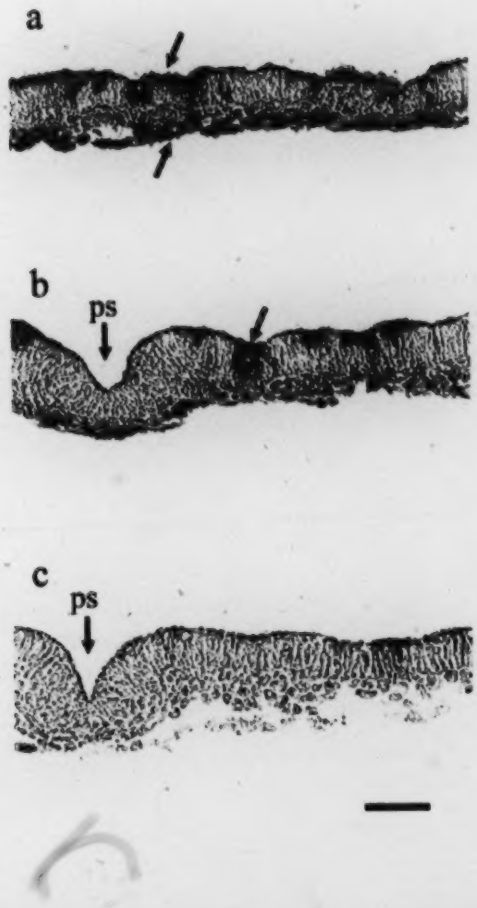


Figure 19. Sections through the primitive streak immunolabeled for paxillin. (a) A section ventro-lateral to the primitive steak showing strong mesodermal and endodermal labeling for paxillin (bottom arrow), and labeling in some epiblast cells (top arrow). (b) A transverse section through the primitive streak showing immunoreactivity in the mesoderm and endoderm cells as they emerge from the primitive streak and epiblast cells ventro-lateral to the primitive streak (arrow). (c) Control section in which the primary antibody was replaced with mouse IgG. Magnification x250; Scale bar = 100μm.

Comparison of paxillin expression *in vivo* and *in vitro*

To evaluate if the transition from *in vivo* conditions to two-dimensional *in vitro* culture conditions is associated with a change in the level of paxillin protein expression, tissue from the three germ layers was dissected from gastrulation stage embryos and either homogenised immediately or grown on fibronectin-coated glass coverslips for 24 hours before homogenisation. Equal amounts of protein from each sample were run on an 8% SDS-polyacrylamide gel and the levels of paxillin protein in each sample were examined by immunoblotting.

Immunoblot analysis using monoclonal anti-paxillin antibody showed a major band in each lane at 68kDa that represented the full-length paxillin protein (Figure 20a). This band was quantified by densitometry (Figure 20b).

Comparison of paxillin levels *in vivo* and *in vitro* showed that there was an overall indication of increased levels of paxillin in each germ layer *in vitro*. (Figure 20b). These differences indicated trends in protein levels the significance of which could not be determined due to the low number of experiments.

Epiblast and endoderm samples from *in vitro* conditions showed an extra band at 46kDa. These were minor bands compared to the band at 68kDa in each lane and probably represent breakdown products of paxillin.

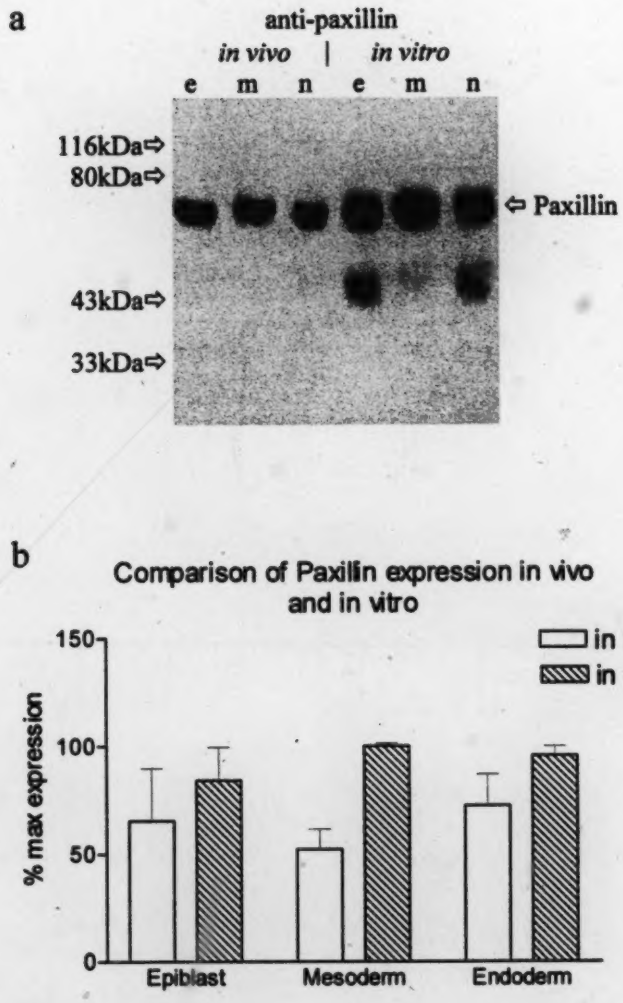


Figure 20. Comparison of paxillin expression levels in the tissues of the stage 5 chick embryo and in cultures grown on fibronectin. (a) Immunoblot showing paxillin protein levels in the epiblast (lanes e), mesoderm (lanes m) and endoderm (lanes n) from tissue dissected directly from each germ layer (in vivo) and from tissue cultured on fibronectin for 24 hours (in vitro). Molecular weight markers are indicated at the left (b) Average level of paxillin expression as determined by densitometry of immunoblots, (n=2). Bars represent the standard error from the mean.

Chapter 5

SIGNALING THROUGH FAK *IN VITRO*

EFFECT OF SUBSTRATUM COMPOSITION *IN VITRO* ON FAK EXPRESSION AND PHOSPHORYLATION[4]

Fibronectin substratum

To examine the effects of substratum composition on FAK expression, cells from each of the germ layers were grown in culture on different substrata for 48 hours, lysed into lysis buffer and the FAK protein and phosphotyrosine levels assessed by immunoblotting.

Cultures grown on a fibronectin substratum showed levels of FAK expression (Figure 21a) very different from those *in vivo* (compare to Figure 10a). Compared to the FAK protein expression pattern *in vivo*, where there was a trend towards highest levels of expression in the epiblast, in cultures grown on fibronectin there was a trend towards higher levels of FAK expression in the endoderm and lower levels of FAK expression in the epiblast, although the levels of FAK expression in each germ layer were not found to be significantly different (Figure 21a).

Nitrocellulose membranes were stripped and reprobed to allow study of the phosphotyrosine levels in these immunoblots. The band at 125kDa (Figure 21b), corresponding to the band on the FAK immunoblot, was quantified in each lane, showing that there was no significant difference between the levels of phosphotyrosine at 125kDa in each sample (Figure 21d).

The corresponding phosphotyrosine bands, at the correct molecular weight, were quantified and related to the amount of FAK present in each sample. Relative to the amount of FAK protein expressed, there was no significant difference in the levels of FAK phosphotyrosine content in any of the cell types grown on fibronectin (Figure 21e).

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[4] *Parts of this chapter have been published in, Ridyard, M. S. and Sanders, E. J. (1998) Cellular phenotypic transformation during early embryogenesis: a role for focal adhesion kinase? Biochem. Cell Biol. 76: 45-58*

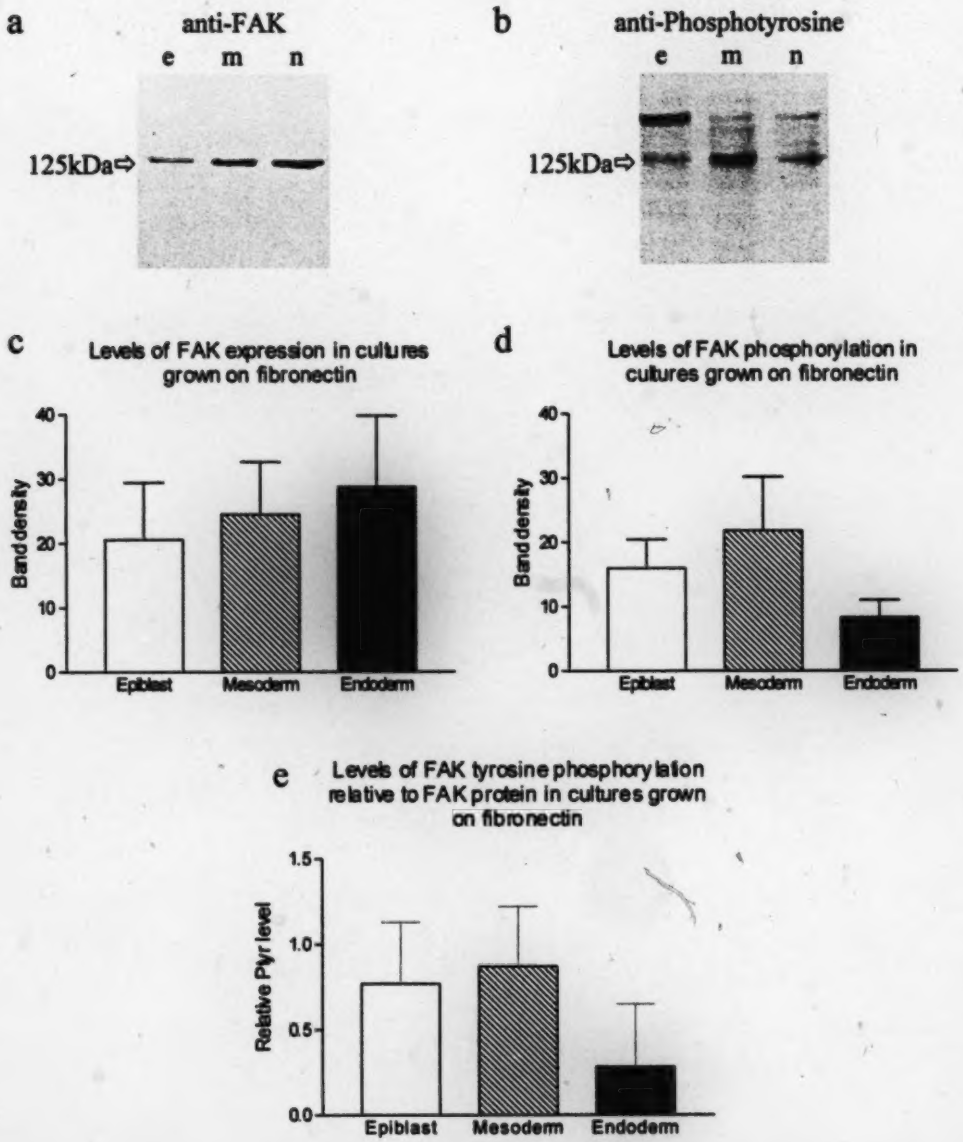


Figure 21. FAK expression and phosphorylation in germ layer cultures grown on fibronectin. (a) epiblast (lane e), mesoderm (lane m), and endoderm (lane n) cultures immunoblotted with monoclonal anti-FAK. (b) The same lanes as shown in (a), but stripped and re-blotted with monoclonal anti-phosphotyrosine. (c) Average densitometric scan of the 125kDa bands in FAK blots (n=4). (d) Average densitometric scan of the 125kDa bands in phosphotyrosine blots (n=2). (e) The levels in (d) divided by the levels in (c), to provide levels of phosphorylation relative to levels of FAK expression.

Laminin substratum

Cells from the three different germ layers, cultured with laminin as a substratum, showed that there was a significant difference in levels of FAK expression between the germ layers. High levels of FAK expression were seen in the endoderm and low levels of FAK expression in the epiblast (Figure 22c).

After stripping and reprobing the immunoblot with anti-phosphotyrosine antibodies, the levels of tyrosine phosphorylation were studied in each sample. Again, similar results were observed in cultures grown on laminin as for cultures grown on fibronectin, with a major band on the immunoblot 125kDa (Figure 22b). Densitometric analysis of the 125kDa bands (Figure 22d) showed that there was no significant difference between the levels of tyrosine phosphorylation at 125kDa in any of the germ layers. Comparing these levels of tyrosine phosphorylation on laminin to the levels of FAK protein expression on laminin (Figure 22e), showed similar results to cultures grown on fibronectin, with no significant difference between the levels of FAK phosphotyrosine in any of the germ layers when grown on a laminin substratum.

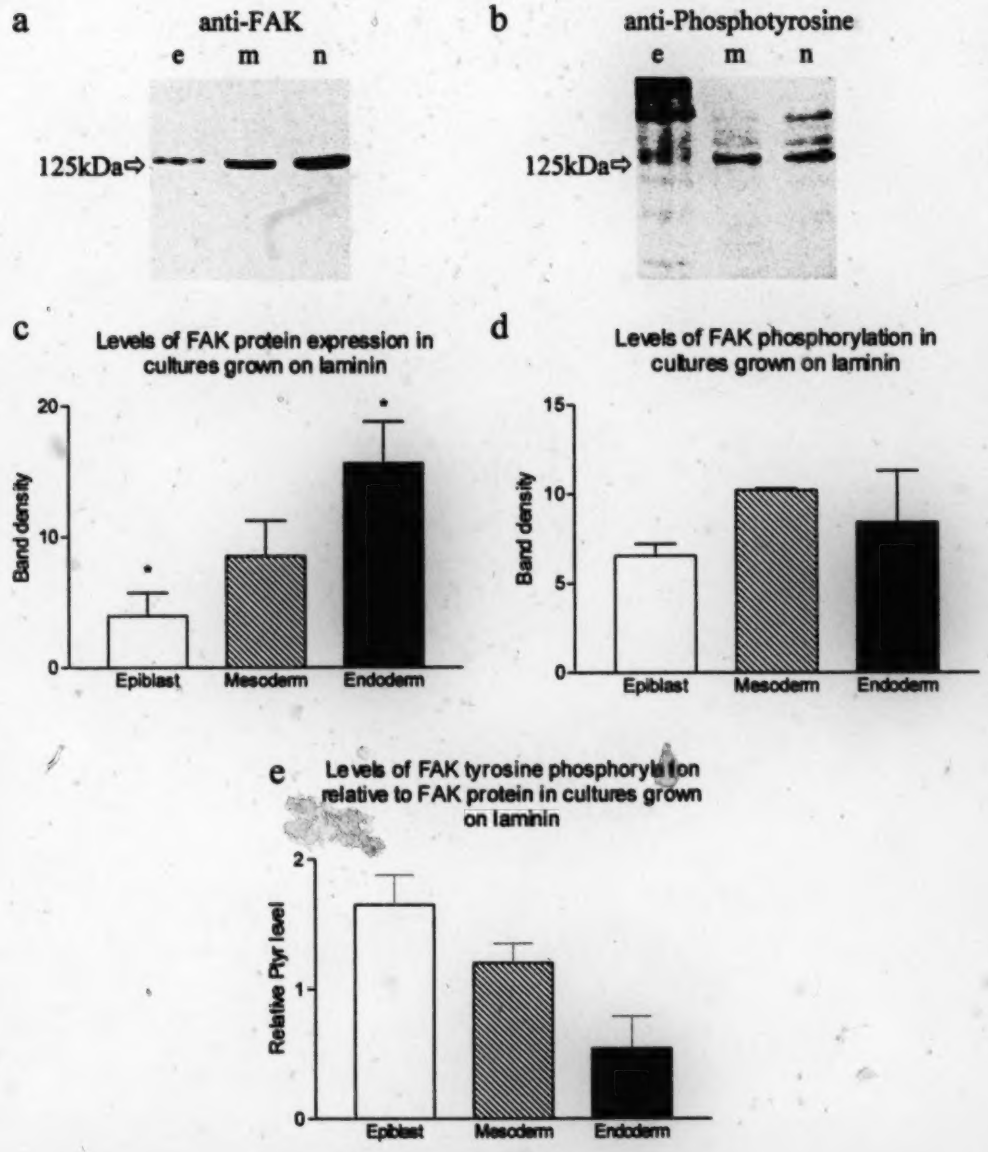


Figure 22. FAK expression and phosphorylation in germ layer cultures grown on laminin. (a) epiblast (lane e), mesoderm (lane m), and endoderm (lane n) cultures immunoblotted with monoclonal anti-FAK. (b) The same lanes as shown in (a), but stripped and re-blotted with monoclonal anti-phosphotyrosine. (c) Average densitometric scan of the 125kDa bands in FAK blots (n=4). In this figure, the epiblast is different from the endoderm at $p<0.05$ (d) Average densitometric scan of the 125kDa bands in phosphotyrosine blots (n=2). (e) The levels in (d) divided by the levels in (c), to provide levels of phosphorylation relative to levels of FAK expression. (*$p<0.05$)

Matrigel substratum

Cells from the three different germ layers were also cultured using growth factor-reduced Matrigel as a substratum. Matrigel is a mixture of several different ECM proteins and provides an *in vitro* environment similar to that encountered by epiblast growing on a basement membrane *in vivo*.

After lysis and SDS-PAGE of the cultures from each germ layer, the pattern of FAK expression was examined by immunoblotting (Figure 23a). The results showed a similar pattern to that seen in both the cultures on fibronectin and laminin substrata. The resulting FAK bands were examined by densitometry and showed that there was no significant difference between the levels of FAK expressed in any of the germ layers when grown on Matrigel (Figure 23c).

The membrane was stripped and the levels of phosphotyrosine were examined (Figure 23b). The band corresponding to FAK at 125kDa was analyzed by densitometry. The result showed no significant differences in the levels of phosphotyrosine between the germ layers relative to each other (Figure 23d). These phosphotyrosine levels were compared to the levels of FAK protein and this showed that there was no significant difference between the levels of FAK phosphotyrosine under these conditions (Figure 23e).

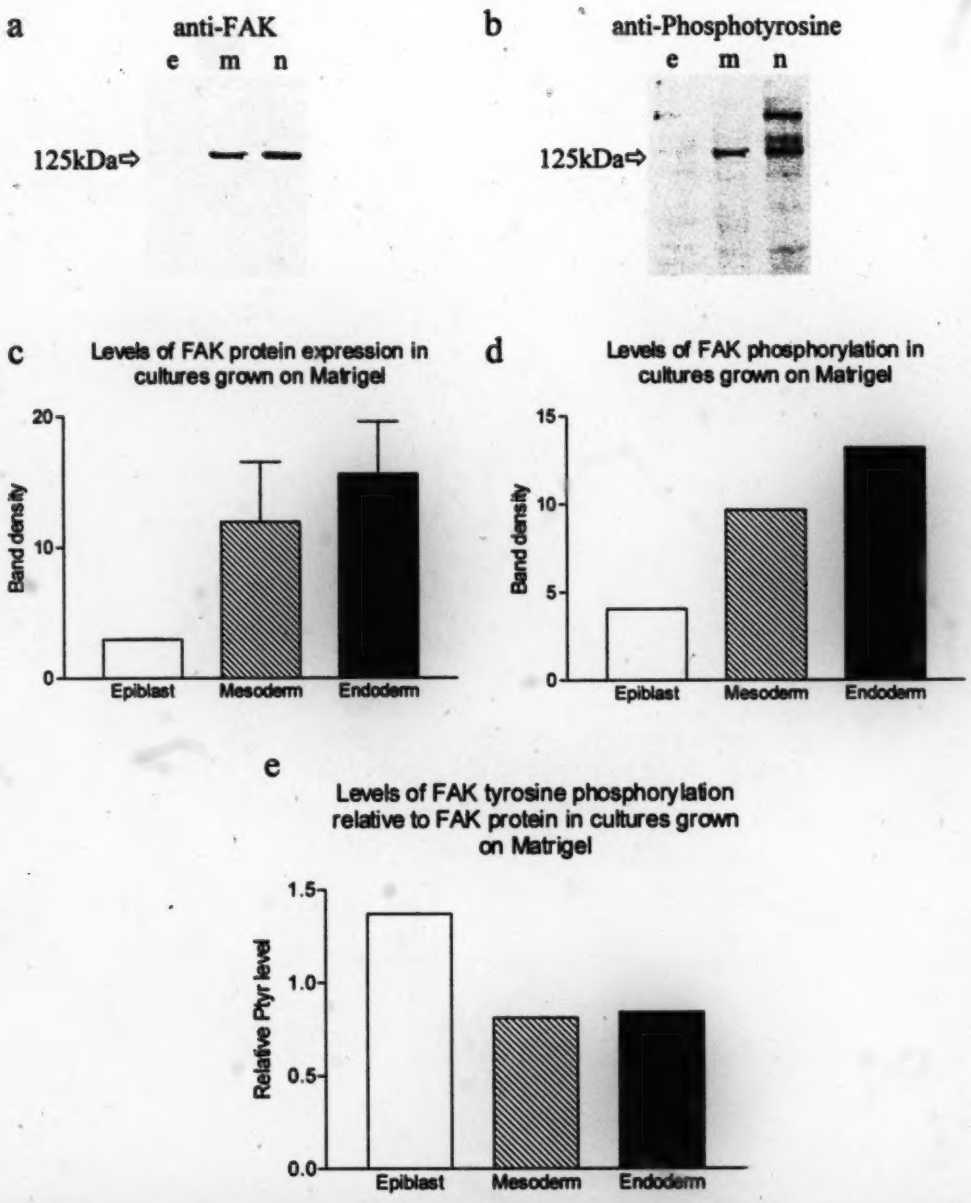


Figure 23. FAK expression and phosphorylation in germ layer cultures grown on Matrigel. (a) epiblast (lane e), mesoderm (lane m), and endoderm (lane n) cultures immunoblotted with monoclonal anti-FAK. (b) The same lanes as shown in (a), but stripped and re-blotted with monoclonal anti-phosphotyrosine. (c) Average densitometric scan of the 125kDa bands in FAK blots (n=4). (d) Average densitometric scan of the 125kDa bands in phosphotyrosine blots (n=2). (e) The levels in (d) divided by the levels in (c), to provide levels of phosphorylation relative to levels of FAK expression.

IMMUNOPRECIPITATION OF FAK UNDER NATIVE CONDITIONS

To identify some of the molecules involved in FAK signal transduction FAK was immunoprecipitated, under non-denaturing conditions, from lysates of whole gastrulation stage embryos or from lysates of gastrulation stage tissues grown in culture. These conditions should allow continued physical association between FAK and other molecules enabling identification of FAK associated molecules.

Three different buffers were used, each with a different non-ionic detergent, to give different condition for solubilisation, allowing immunoprecipitation of protein complexes. The results were the same with each buffer.

Analysis of FAK immunoprecipitations by immunoblotting showed FAK was successfully immunoprecipitated from both whole embryo and cell culture samples (Figures 24a, lanes 2 and 4). The majority of FAK in each sample was immunoprecipitated and very little FAK was left in the lysate (Figures 24a, lanes 3 and 5).

The immunoblot was stripped and reprobed with anti-paxillin antibodies showing that no paxillin was immunoprecipitated with FAK in either sample (Figures 24b, lanes 2 and 4). However, paxillin is present in the lysate of each sample (Figures 24b, lanes 3 and 5).

The immunoblot was stripped and reprobed with anti-Src antibodies showing that no Src was immunoprecipitated with FAK in either sample (Figures 24c, lanes 2 and 4). Src is present in the lysate of each sample, however (Figures 24c, lanes 3 and 5).

None of the immunoblots show an association between FAK and paxillin or Src under the conditions used for immunoprecipitation.

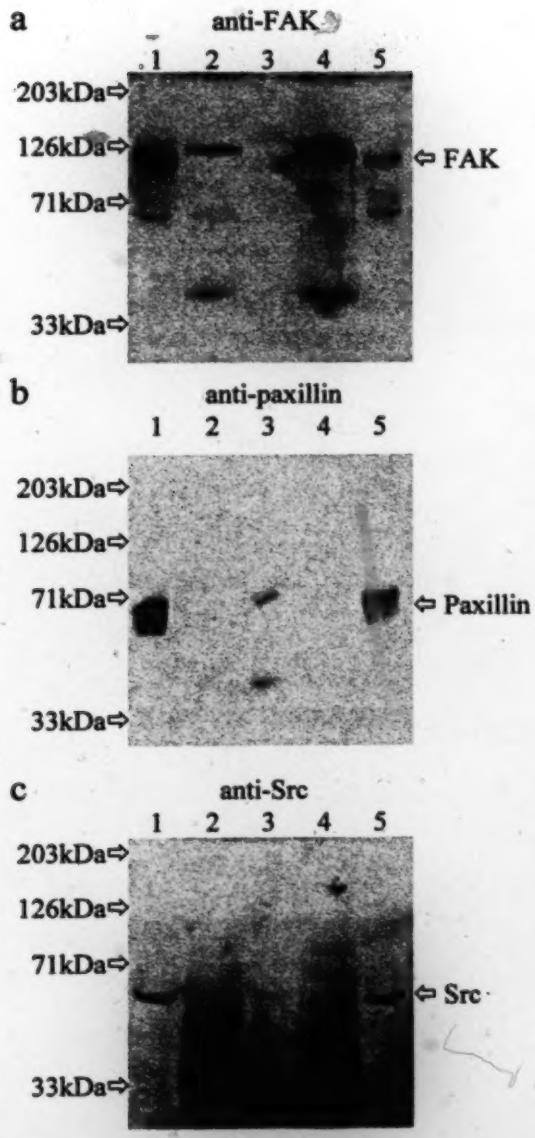


Figure 24. Immunoprecipitation of FAK and associated proteins. (a) Immunoblot for FAK on whole embryo lysate (lane 1), cell cultures immunoprecipitated with anti-FAK (lane 2), the supernatant from cell cultures after immunoprecipitation with anti-FAK (lane 3) whole embryo lysate immunoprecipitated with anti-FAK (lane 4), and the supernatant from whole embryo lysate after immunoprecipitation with anti-FAK (lane 5). (b) The same blot as in (a), stripped and reprobed with antibodies against paxillin. (c) The same blot as in (a), stripped and reprobed with antibodies against Src. Molecular weight markers are indicated to the left.

Chapter 6

EFFECTS OF REDUCED FAK PROTEIN EXPRESSION AFTER ANTISENSE OLIGONUCLEOTIDE TREATMENT

EFFECT OF OLIGONUCLEOTIDE TREATMENT ON PROTEIN EXPRESSION AND LEVELS OF APOPTOSIS.

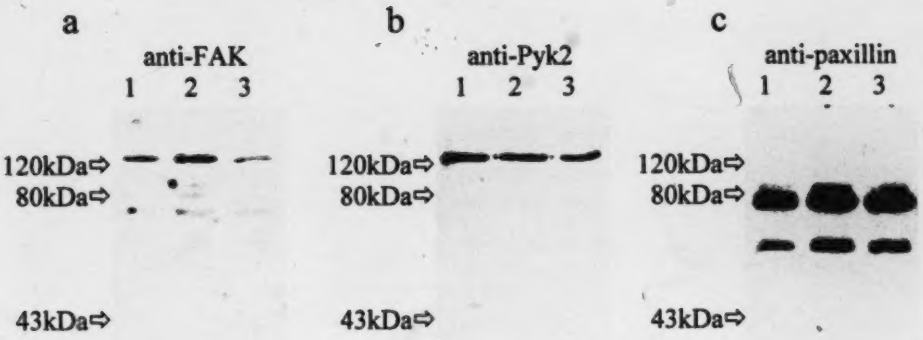
To analyse the function of FAK in chick embryo cells the level of FAK protein expression was specifically reduced by antisense oligonucleotide-treatment.

Epiblast cells from gastrulation stage embryos were grown on fibronectin coated coverslips for 24 hours before replacement of the media with oligonucleotide-containing media. Oligonucleotides were successfully introduced into the cells and visualisation of biotinylated oligonucleotides using FITC-conjugated streptavidin showed the presence of oligonucleotides in a high percentage of nuclei in the culture (Figure 25).

Immunoblot analysis showed the effect of antisense oligonucleotides on protein expression was specific for FAK (Figure 26a). Repeated immunoblots showed antisense oligonucleotides reduced FAK expression to 30% that of untreated epiblast cultures or epiblast cultures treated with sense oligonucleotides (Figure 26d). Both Pyk2 and paxillin expression were unaffected by addition of either sense or antisense oligonucleotides (Figure 26c,d).

To determine if the treatment of epiblast cells with antisense oligonucleotides to FAK affected the levels of apoptosis in cultured cells, the number of apoptotic nuclei in each culture were counted (Figure 27). Apoptotic nuclei were visible with DAPI staining as condensed spots that have been shown to correlate with TUNEL labeling of apoptotic nuclei (see Figure 28). None of the cultures showed particularly high levels of apoptosis. The sense oligonucleotide-treated cultures showed similar levels of apoptosis to the untreated cultures and the antisense oligonucleotide-treated cultures showed a slight increase although it was not found to be significantly different from the other conditions (Figure 27d).

Figure 25. Localisation of oligonucleotides after treatment of cultured cells. Biotinylated oligonucleotides, visualised with FITC-conjugated streptavidin, are visible in the nucleus of (a) sense oligonucleotide and (b) antisense oligonucleotide-treated cultures but not (c) untreated or (d) cultures treated only with cytofectin. Scale bar=50μM.



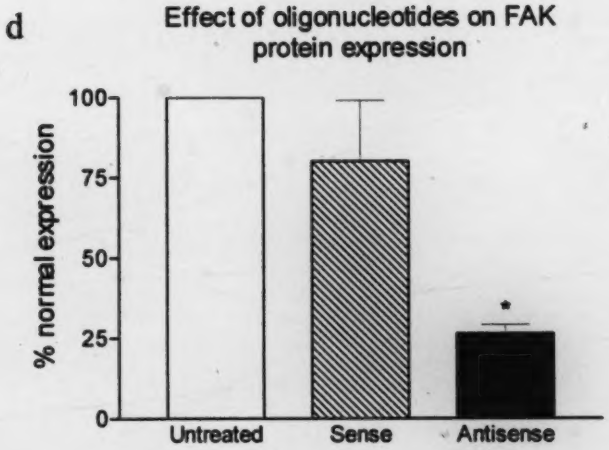
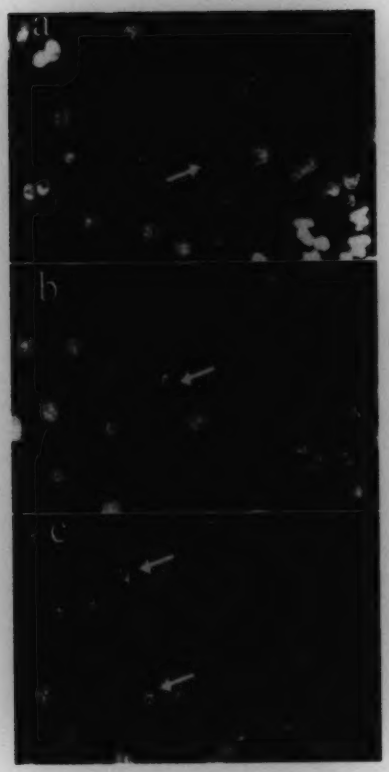


Figure 26. Effect of oligonucleotides on protein expression. Untreated (lanes 1), sense oligonucleotide-treated (lanes 2) or antisense oligonucleotide-treated (lanes 3) epiblast cultures immunoblotted for (a) FAK, (b) Pyk2 and (c) paxillin. (d) Quantification of FAK immunoblots by densitometry shows a significant reduction of FAK expression to 30% the level of untreated or sense oligonucleotide-treated cultures (n=3; *$p<0.05$).



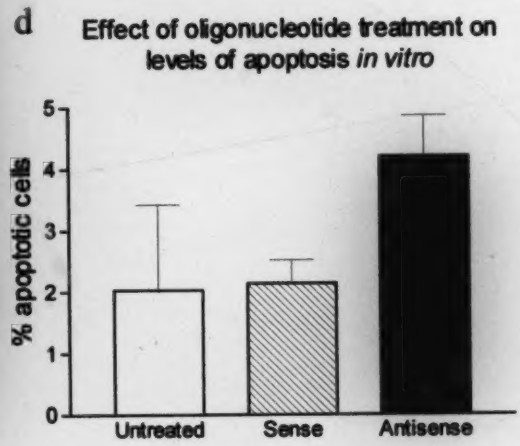


Figure 27. Effect of oligonucleotides on levels of apoptosis in culture. (a) DAPI labeled nuclei in untreated epiblast cultures showing condensed apoptotic nuclei (arrow). (b) DAPI labeled nuclei in sense oligonucleotide-treated cultures showing condensed apoptotic nuclei (arrow). (c) DAPI labeled nuclei in antisense oligonucleotide-treated epiblast cultures showing condensed apoptotic nuclei (arrows). (d) Antisense oligonucleotide-treated epiblast cultures show a rise in the percentage of apoptotic nuclei compared to untreated or sense oligonucleotide-treated cultures. Levels are not significantly different (n=3).

EFFECTS OF SERUM LEVEL ON FAK AND PYK2 EXPRESSION AND APOPTOSIS

To determine whether there was a correlation between FAK expression and levels of cell death, cultures of epiblast tissue were grown for 24 hours on fibronectin-coated coverslips before 24 hours of treatment in medium containing either 10% or 0% serum. Control cultures were grown in medium supplemented with 10% serum for the full 48 hours.

Cultures were examined by double labeling with TUNEL, for identification of apoptotic cells, and DAPI, for visualisation of all nuclei. After serum starvation for 24 hours fragmenting apoptotic nuclei were visible in the cultures (Figure 28a, arrows). These nuclei were also visible as condensed nuclei using the DAPI label for DNA (Figure 28b, arrows). Control cultures that were grown for 48 hours in medium containing 10% serum showed only background labeling in some cells at the periphery of the culture (Figure 28c).

Quantification of TUNEL labeled nuclei, as a percentage of total nuclei, showed that in control cultures approximately 1% of the cells were apoptotic. Cultures that had been serum starved for 24 hours showed an increase from the control level with approximately 5% of the cells being apoptotic (Figure 28e).

Expression levels of FAK (Figure 29a) and Pyk2 protein (Figure 29b) in control and serum starved cultures were examined by immunobloting. Quantification of the immunoblots by densitometry showed no significant difference between the levels of FAK (Figure 29c) or Pyk2 (Figure 29d) expression in control and serum starved cultures.

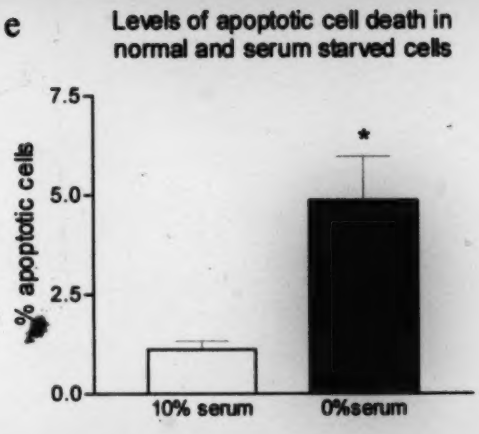


Figure 28. Detection of apoptotic cells in cultures supplemented with 0% or 10% serum. (a,b) Epiblast cultures grown for 24 hours in medium with 10% serum then for 24 hours in medium with 0% serum, arrows indicate apoptotic cells. (c,d) Epiblast cultures grown for 48 hours in medium with 10% serum. Cells are double-labeled with TUNEL (a,c) for visualisation of apoptotic nuclei, and DAPI (b,d) for visualisation of all nuclei. (e) Quantification of the number of apoptotic nuclei, as a percentage of total nuclei, in 10% serum and 0% serum cultures. (* $p<0.05$, $n=5$).

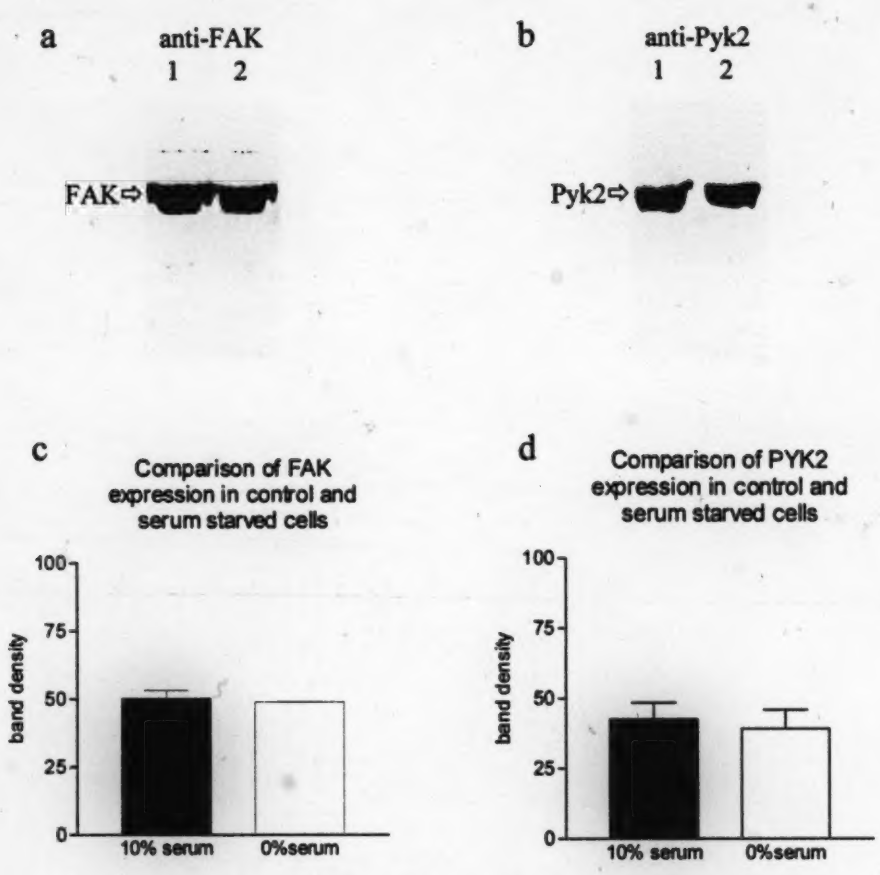
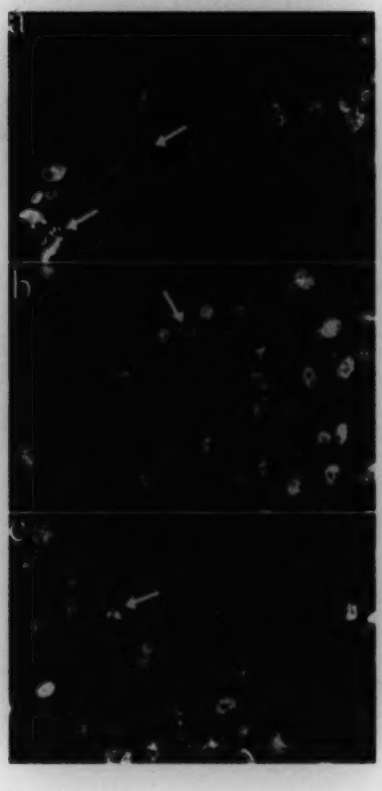


Figure 29. Analysis of FAK and Pyk2 expression in cells cultured with 0% or 10% serum. (a) Epiblast cultures grown for 48 hours in medium with 10% serum (Lane 1) or epiblast cultures grown for 24 hours in medium with 10% serum then for 24 hours in medium with 0% serum (Lane 2), immunoblotted with antibodies against FAK. (b) The same membrane as in (a) stripped and reprobed with antibodies against Pyk2. (c) Average band densities of bands form FAK blots determined by densitometric analysis (n=2). (d) Average band densities of bands form Pyk2 blots determined by densitometric analysis (n=2).

EFFECT OF OLIGONUCLEOTIDE TREATMENT AND SERUM STARVATION ON LEVELS APOPTOSIS

Cultures of epiblast were grown on fibronectin-coated coverslips for 24 hours in media with 10% serum before 24 hours of treatment in media with either a) 0% serum and 20μM sense oligonucleotide b) 0% serum and 20μM antisense oligonucleotide or c) 0% serum with no oligonucleotide.

To determine if the treatment of cells with antisense oligonucleotides to FAK affected the levels of apoptosis in cultured cells, the number of apoptotic nuclei in each culture were counted (Figure 30). None of the cultures showed high levels of apoptosis. All cultures showed similar levels of apoptosis under each condition with no significant increase in apoptosis observed when serum starved cultures were treated with antisense oligonucleotides (Figure 30d).



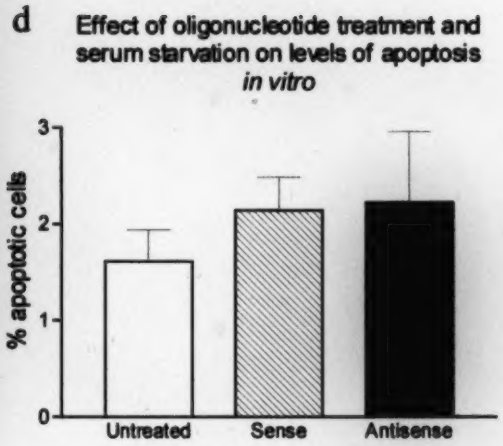


Figure 30. Effect of oligonucleotide and serum starvation on levels of apoptosis. (a) DAPI labeled nuclei in serum starved cultures showing condensed apoptotic nuclei (arrows). (b) DAPI labeled nuclei in sense oligonucleotide-treated and serum starved cultures showing condensed apoptotic nuclei (arrow). (c) DAPI labeled nuclei in antisense oligonucleotide-treated and serum starved cultures showing condensed apoptotic nuclei (arrow). (d) Antisense oligonucleotide-treated cultured cells show similar levels of apoptotic nuclei compared to untreated or sense oligonucleotide-treated cultures under serum starved conditions (n=3).

EFFECT OF OLIGONUCLEOTIDE TREATMENT ON FAK, PAXILLIN AND ACTIN LOCALISATION

Epiblast cultures were grown on fibronectin-coated coverslips for 24 hours in media with 10% serum before 24 hours of treatment in media with either a) 0% serum and 20μM sense oligonucleotide b) 0% serum and 20μM antisense oligonucleotide or c) 0% serum with no oligonucleotide.

Immunolabeling for FAK revealed a similar pattern of localisation in the untreated (Figure 31a) and sense oligonucleotide-treated cells (Figure 31c). In these cells FAK localised to focal adhesion sites and also to the nucleus. In comparison, FAK showed little localisation to the nucleus in antisense oligonucleotide-treated cells and labeling for FAK in focal adhesions appeared reduced (Figure 31e).

Immunolabeling for paxillin showed colocalisation of paxillin with FAK in focal adhesions in both the untreated (Figure 31b) and sense oligonucleotide-treated cultures (Figure 31d). In contrast to FAK, paxillin immunolabeling remained strong in antisense oligonucleotide-treated cultures (Figure 31f), localising to focal adhesions.

Although paxillin immunolabeling showed that focal adhesions were still present in the antisense oligonucleotide-treated cultures, the distribution of focal adhesions is different. Untreated and sense oligonucleotide-treated cultures both show a distribution of focal adhesions throughout the cell compared to antisense oligonucleotide-treated cells which show focal adhesions only around the periphery of the cell (compare Figures 31b,d with Figure 31f).

Examination of the actin cytoskeleton showed that the changes in focal adhesion distribution also correlated with changes in arrangement the actin cytoskeleton. In untreated (Figure 32a) and sense oligonucleotide-treated cultures (Figure 32b) there were long stress fibres present throughout the cell that were oriented in a particular direction suggesting a polarised, migratory phenotype. Cultures treated with antisense oligonucleotides to FAK showed very few stress fibres present in the cell, generally showing a less polarised morphology with bundles of actin around the periphery of the cell (Figure 32c arrows).

Analysis of cell shape shows that there are differences in the morphology of the

untreated, sense and antisense oligonucleotide-treated cells. Measurements of cell area show that the antisense oligonucleotide-treated cells were spread over a significantly lower area than cells in untreated or sense oligonucleotide-treated cultures (Table 1). Measurements of aspect ratio, the long axis divided by the short axis of the cell to give an indication of the level of polarisation of cell shape, shows that the antisense oligonucleotide-treated cultures have a value nearer to 1 than either the untreated or sense oligonucleotide-treated cultures, both of which have a similar value, indicating similar levels of polarised cell shape (Table 1).

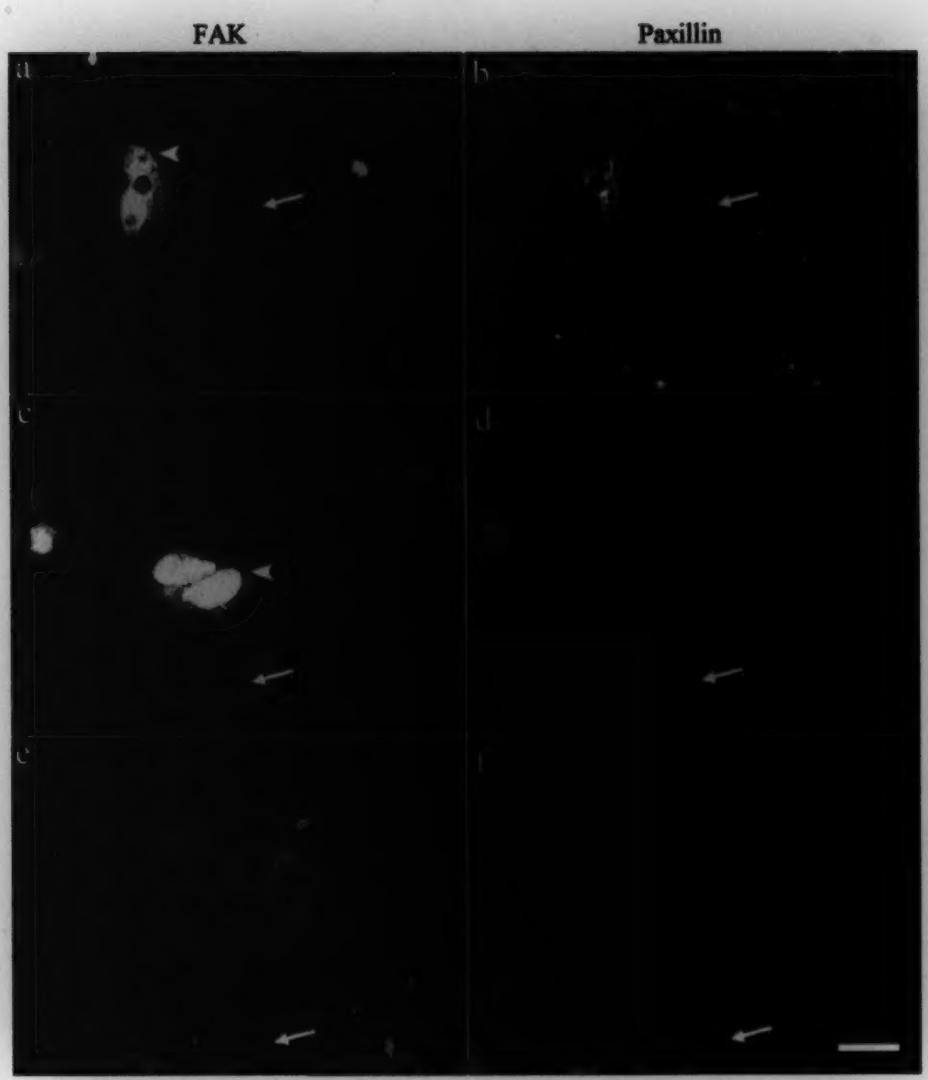


Figure 31. Effect of oligonucleotides on the localisation of FAK and paxillin. FAK (a,c,e) and paxillin (b,d,f) immunolabeling in cultured cells. Cells were treated with regular medium (a,b) medium with sense oligonucleotide (c,d) or medium with antisense oligonucleotide (e,f). FAK and paxillin co-localise to focal adhesions (arrows) although the labeling for FAK in antisense oligonucleotide-treated cultures is reduced. FAK also shows nuclear labeling (arrowheads) which is also reduced in antisense oligonucleotide-treated cultures. Scale bar=25μM.

Figure 32. Effect of oligonucleotides on the actin cytoskeleton. Cultured cells showing the actin cytoskeleton labeled with rhodamine conjugated phalloidin. (a) Cells grown in regular medium show stress fibres throughout the cell. (b) Cells grown with 20μM sense oligonucleotide show similar pattern of stress fibres throughout the cell. (c) Cells grown with 20μM antisense oligonucleotide show bundles of actin around the periphery of the cell (arrows). Scale bar=50μM.

	Untreated	Sense oligonucleotide-treated	Antisense oligonucleotide-treated
Cell area	32.74 ± 1.88	47.05 ± 6.23	22.32 ± 2.75*
Aspect ratio	1.67 ± 0.11	1.71 ± 0.17	1.33 ± 0.08

Table 1. Effect of oligonucleotides on cell area and polarisation. Measurements of cell area show significantly lower values in the antisense oligonucleotide-treated cultures compared to untreated or sense oligonucleotide-treated cultures. Aspect ratio values are measurements of the long axis of the cell divided by the short axis. For aspect ratio, values closer to 1 indicate rounder cells. Values in the table are calculated on an arbitrary scale and represent mean ± S.E.M. ($n=10$; $*p<0.05$).

Chapter 7

DISCUSSION

DISCUSSION

The results presented in this work provide evidence that expression of FAK, Pyk2 and paxillin in embryonic chick cells is spatially regulated during gastrulation. Further evidence shows that tissue specific levels of FAK expression can be influenced by composition of the ECM and that FAK may be involved in signal transduction pathways that regulate the cell cytoskeleton and possibly cell migration during early development.

During embryonic development cells receive cues from their environment which influence morphogenesis. These cues may come from a variety of sources such as interaction with growth factors, from cell-cell interaction, or as a result of binding to ECM proteins. These events can elicit the activation of signaling pathways in the cells that can ultimately affect cell fate by acting on gene expression.

Integrins are the main family of cell surface receptors responsible for cell attachment to the ECM, and integrin adhesion has been shown to modulate several aspects of cell behavior, including proliferation, migration, invasion, matrix assembly, and programmed cell death (Simon and Burridge 1994). The protein tyrosine kinase FAK is activated on integrin-ligand binding and forms part of the signaling complex associated with integrins at focal adhesion sites with the *in vitro* substratum. FAK may therefore be a mediator of the integrin-associated signals during development that co-ordinate morphogenesis and cell migration (Cary et al., 1996; Gilmore and Romer 1996), through interaction of cells with the ECM. Several previous studies have lead to this conclusion, including reports of developmentally regulated FAK expression in the embryos of the mouse (Polte et al., 1994), chick (Turner et al., 1993), and *Xenopus laevis* (Hens and DeSimone 1995; Zhang et al., 1995a). Further, targeted mutation of FAK in the mouse embryo has been shown to result in developmental failure at mid-gastrulation with an overall phenotype suggesting a defect in mesoderm morphogenesis (Furuta et al., 1995).

The model used for studies in this work is the gastrulating chick embryo. Gastrulation is characterised by the ingression of the epithelial epiblast cells through the primitive streak, with an accompanying phenotypic transformation into the fibroblast-like mesoderm cells. This ingression occurs through a break in the basement membrane of the epiblast (Sanders 1984). The immunoreactivity of FAK in the embryo indicates that the

epiblast expresses high levels of FAK, especially at the more rostral levels, but that this is abruptly down regulated as the epiblast cells approach and enter the primitive streak through the disrupted basement membrane. This change in expression therefore correlates with a region in which the substratum and the integrin engagement of the transforming cells also change. The down-regulation of FAK in the epiblast at the primitive streak also correlates with reduced cell proliferation in this region (Sanders et al., 1993), as it does in some other tissues (Tremblay et al., 1996). A recent study has also shown that FAK is involved in cell cycle regulation by integrins, through downstream regulation of D1 and p21 (Zhao et al., 1998). Both FAK expression and proliferation rate may be related to the changes in cell adhesion in the primitive streak region.

Depending on the rostro-caudal level within the embryo, the resulting mesoderm cells may, or may not, transiently re-express FAK before leaving the vicinity of the streak. The fibronectin-rich ECM encountered by the mesoderm cells as they emerge from the primitive streak (Sanders 1982) may be a factor in the increase in FAK expression since fibronectin is an FAK activating substratum (Hanks et al., 1992). A similar expression pattern to the mesoderm layer is observed for the endoderm layer.

Since FAK is localised in focal adhesions in cultured cells the question arises as to the subcellular localisation of FAK in the embryo since embryonic tissues are not known to have focal adhesions. The immunocytochemical results suggest that, while it is widely expressed in the epithelial epiblast cells, FAK is particularly associated with the apical cytoplasm. The ultrastructural immunocytochemistry for FAK confirms the apical localisation, but does not provide evidence that it associates with specialised cell-cell junctions in this region. Previous investigators have also described this apical localisation in epithelia (Tani et al., 1996), and have speculated on the basis of light microscopy that the immunoreactivity is associated with intercellular junctions (Baker et al., 1994; Tani et al., 1996), but the results shown here provide no evidence for this association. The adherens junction is a cell-to-cell adhesion found in the apico-lateral membrane where cadherins associate with the actin cytoskeleton (Yap et al., 1997). The clustering of microfilaments in this junction is analogous to the focal adhesion but the ultrastuctural studies presented here show no evidence that FAK is associated with these junctions.

Integrin engagement to the underlying basement membrane is occurring at the basal surface of the cells, and FAK associated with this engagement would therefore be expected to be in the basal cytoplasm of the cells. The finding that FAK predominates in the apical cytoplasm suggests there may be a spatial separation of FAK and integrins in these cells *in vivo*, in contrast to the organisation *in vitro*. *In vivo*, it is therefore possible that FAK is only indirectly associated with integrin-mediated signaling pathways, and is not physically organised with integrins in focal adhesion-like structures.

There are numerous studies showing results such as FAK phosphorylation independent of focal adhesion formation (Lyman et al., 1997), phosphorylation of mutant FAK that is mislocalised (Zhao et al., 1998) and phosphorylation of FAK lacking the FAT sequence, in attached cells (Hildebrand et al., 1993). These results not only dissociate the formation of the focal adhesion structures from the activation of FAK, but also show that FAK can be phosphorylated in response to adhesion without itself being localised to focal adhesions. This supports the possibility that FAK may not need to be directly associated with integrin adhesive sites in order to mediate integrin signaling. If FAK is not physically associated with integrins in the embryo then there are presumably other signaling molecules upstream of FAK that mediate signaling between integrins and FAK. It has been noted that PKC is activated upstream of FAK in Chinese hamster ovary cells (Vuori and Ruoslahti 1993). PKC did not appear to act directly on FAK but rather through indirect means, possibly through changes in the cytoskeleton. If FAK is still associated with the cytoskeleton in the chick embryo then regulation of the cytoskeleton by integrin adhesion may influence FAK activity and provide a means of FAK regulation by integrins.

The high level of FAK noted in the epiblast of sectioned embryos was paralleled by relatively high expression of phosphorylated FAK in the immunoblotted samples of this tissue. The mesoderm and endoderm showed reduced levels of FAK expression compared to the epiblast but there were only slight variations in the levels of tyrosine phosphorylation between each germ layer that were not found to be significantly different. Although the level of FAK protein appears to be reduced as the cells transform at the primitive streak, the FAK that is expressed in each germ layer appears to have a similar level of activation.

Immunoblotting results also confirmed the reduction in the level of FAK in the primitive streak region seen in the sectioned embryos, with FAK expression levels in this tissue between that of the epiblast and mesoderm. The primitive streak represents an area of phenotypic transition between the epiblast and underlying mesoderm and endoderm and the levels of FAK expressed in the primitive streak reflect the mixed population of cells in this region. These changing FAK expression patterns with differentiation suggest that there is spatiotemporal regulation of FAK protein within the gastrulating embryo, supporting a role for this PTK in signaling during early development.

Because the substrata encountered by cells in the gastrulating embryo change as the epiblast cells ingress and transform, it was of interest to see if the substratum composition in the embryo could be influencing FAK expression. While the epiblast cells attach to their underlying basement membrane, the mesoderm cells, having traversed the primitive streak encounter a fibronectin-rich ECM (Sanders 1982) which influences their spreading and motility (Sanders 1980). Fibronectin, in particular, has previously been shown to influence the tyrosine-phosphorylated state of FAK in cultured cells (Hanks et al., 1992).

Culture of gastrulation stage cells on the three different substrata, fibronectin, laminin and Matrigel resulted in tissue specific effects on the levels of FAK protein expression. There did not appear to be any significant difference in the expression of FAK levels between the germ layers when cells were grown on either fibronectin or Matrigel substrata. In contrast there was a significant difference between the levels of FAK in endoderm and epiblast cells grown on a laminin substratum. This shows that there is a tissue specific effect on FAK expression when cells are grown on laminin but not fibronectin or Matrigel, possibly due to the different integrins which would be used to engage the different substrata having different effects on cell behavior. It was notable that the levels of FAK tyrosine phosphorylation did not appear to change when cells were grown on different substrata. This was similar to the situation found in the embryo where there was no significant difference in FAK tyrosine phosphorylation between the germ layers. This may reflect the importance of regulating FAK protein levels rather than phosphorylation as a means of regulating signaling pathways in these cells.

It was not the purpose of the *in vitro* experiments to duplicate the *in vivo* levels of FAK expression and tyrosine phosphorylation, which presumably depend on interactions between the tissues, complex ECM and growth factors. However, these results do show that the substratum, and the resulting integrin engagement of these cells, is capable of influencing FAK expression in a tissue specific manner, although the relative levels of tyrosine phosphorylation of this molecule do not appear to be affected by substratum composition. These results also suggest that this influence of the changing extracellular matrix on FAK may be related to the onset of mesodermal cell invasion through the basement membrane and the subsequent mesodermal cell migration, in an analogous way to that proposed for several tumour cell types (Matsumoto et al., 1994; Akasaka et al., 1995; Owens et al., 1995).

All cell types from the gastrulating embryo express FAK in culture. Staining was localised to focal adhesions as identified by IRM in all cells as expected, but mesoderm cells also showed staining over broad patches of lamellipodia, as well as in focal adhesions. By IRM, these patches of immunoreactivity corresponded to areas described as "close contacts", which are regions of the cell separated from the substratum by a distance of approximately 30nm, compared to focal adhesions at 10 – 15nm separation (Verschueren 1985). Close contacts are areas of relatively weak adhesion between the cell and the substratum and are most frequently found in highly motile cells. Microfilaments and another focal adhesion protein, α-actinin, have been found in close contacts (Heath and Dunn 1978; Chen and Singer 1982). There is some dispute as to whether the interpretation of the IRM image in the lamellipodium can be misleading due to the thinness of the cytoplasm and possible interference from the dorsal cell membrane (Gingell and Todd 1979). Since most of the immunoreactivity that appears as large patches is found in the lamellipodia, this makes it difficult to conclude whether the immunoreactivity correlates with close contacts or if they actually represent very large focal adhesion structures. If these are close contacts, the possible presence of FAK, along with the α-actinin, suggests that these contacts may have the capability to associate with the cytoskeleton and possibly activate "outside-in" tyrosine kinase signaling pathways, similar to that occurring at focal adhesions.

An unusual feature of the immunolabeling for FAK in cultured cells was an apparent nuclear localisation of FAK. Other investigators have noted that another focal adhesion protein, zyxin, has been shown to translocate from the cell membrane to the nucleus (Nix and Beckerle 1997). In the case of zyxin, it has been proposed that shuttling could be a means of communication from sites of cell adhesion to the nucleus. Unlike zyxin, which possesses DNA binding LIM domains and a nuclear export signal (Nix and Beckerle 1997), FAK does not have any known nuclear targeting sequence and any nuclear localisation is likely to be through interaction with an intermediary protein. In this context it is interesting to note that the PTK, Src, an FAK binding protein (Thomas et al., 1998), can be found in focal adhesions and in the nucleus in cultured cells (Moszczynska and Opas 1993). FAK has also been localised to the nucleus in other studies (Fernandez-Valle et al., 1998). The function that FAK may have in the nucleus is not known although it may still be involved in the regulation of actin microfilament networks. Actin itself has been found to be present within the nucleus in both polymerised and unpolymerised forms (Rubin et al., 1978). When the function of nuclear actin is disrupted, by introduction of actin-binding antibodies into the nucleus, there is an inhibition of transcription suggesting that actin is important in the organisation of the chromosome during transcription of the genome (Scheer et al., 1984). How actin is involved in this process is not clear, but FAK may be involved in formation of an actin based signaling complex in the nucleus at sites of transcription, regulating transcription through phosphorylation of transcription factors. The localisation of FAK in the nucleus may also be important in the cells' ability to respond to transmission of signals through the cell as a result of cellular tensegrity. The mechanical stimulation of the nucleus by the cytoskeleton may lead to changes in nuclear function (Ingber 1997). These stimuli may lead to changes in the organisation of the actin present within the nucleus and this may regulate the activity of FAK within the nucleus. Reduction of FAK protein expression by treatment of chick embryo cells with antisense oligonucleotides to FAK (see below) showed that after treatment the cells had a reduced level of FAK immunoreactivity in the nucleus. This would suggest the localisation of FAK within the nucleus is not an artifact.

It was not possible to detect Pyk2 localisation to focal adhesions *in vitro*, however,

nuclear localisation of Pyk2 similar to that seen for FAK was observed. Pyk2 showed nuclear localisation only in selected cells, the significance of which is unclear, but it may reflect a shuttling of Pyk2 between the nucleus and cytoplasm, perhaps at different stages of the cell cycle or cell adhesion site formation. The function of Pyk2 in the nucleus is unknown although its homology to FAK would suggest a similar function, perhaps in the regulation of transcription or regulation of nuclear trafficking through association with the actin present in the nucleus. Both FAK and Pyk2 bind paxillin (Hildebrand et al., 1995; Gismondi et al., 1997) and have a high degree of homology in the focal adhesion targeting domain (Hildebrand et al., 1993; Sasaki et al., 1995). Despite their similar structure these results, along with other studies showing cytoplasmic localisation of Pyk2 (Zheng et al., 1998), show FAK and Pyk2 are regulated differently, since only FAK shows focal adhesion localisation. The possible nuclear localisation of FAK and Pyk2 shows a degree of overlap in their localisation and possibly reflects an overlap in their function within the cells.

FAK and Pyk2 share overall structural homology but have sequence differences in the N and C terminal non-catalytic domains (Sasaki et al., 1995). The differences observed in signaling activity (Schaller and Sasaki 1997) and localisation of FAK and Pyk2 are presumably due to these differences.

The localisation of Pyk2 in other cell types *in vitro* is somewhat unclear. Pyk2 has been localised to sites of cell-to-cell (Sasaki et al., 1995) as well as to sites of cell-to-ECM adhesion such as the sealing zone in osteoclasts (Lakkakorpi et al., 1999) and focal adhesion like structures in megakaryocytes (Li et al., 1996). Studies in chick embryo lines and rat fibroblast cells show a small percentage of Pyk2 is localised in focal adhesions (Schaller and Sasaki 1997; Matsuya et al., 1998).

The diversity of intracellular localisation of Pyk2 correlates with the differing reports on stimuli that activate Pyk2. Like FAK, Pyk2 is activated by integrin engagement in some cell lines (Astier et al., 1997a), however, conflicting reports show little effect of integrin adhesion on Pyk2 activation but marked activation by soluble stimuli (Zheng et al., 1998). The varied reports of Pyk2 localisation and activation may reflect the somewhat overlapping functions of Pyk2 and FAK in some cells lines (Schaller and Sasaki 1997; Sieg

et al., 1998).

There did not appear to be any specific localisation of Pyk2 with in the embryo as judged by immunocytochemistry. The immunoreactivity throughout the embryo was very low, which correlated with the low levels of Pyk2 expression seen on immunoblots (see below). There did appear to be a gradient of Pyk2 expression within the embryo, however, with higher expression in the anterior region of the embryo and lower expression in the posterior region. The anterior of the embryo is at a slightly later stage of development than the posterior during early development suggesting Pyk2 may be regulated during development, with increasing expression at later stages. Pyk2 is possibly expressed at higher levels in neuronal cell types (Mitaka et al., 1997) where it may be involved in regulation of ion channels (Lev et al., 1995). Since the gastrulating embryo does not have differentiated neuronal cells this may be one reason for the lower expression of Pyk2 at this stage of development.

Paxillin is a 68kDa cytoplasmic protein with multiple protein interaction domains allowing it to act as an adapter protein (Turner and Miller 1994). FAK has been shown to associate with paxillin *in vitro* (Hildebrand et al., 1995), and paxillin has been suggested to be a possible mediator for FAK localisation in focal adhesions. These results show that both of these proteins localise to focal adhesions as well as broader areas that may represent close contacts in chick embryo cells. Unlike FAK, however, paxillin showed no nuclear localisation.

In the embryo there was some similarity between the localisation of paxillin and FAK, with paxillin showing some apical localisation in the epiblast tissue. This may reflect the fact that FAK and paxillin are involved in the same signaling pathways *in vivo*, requiring their co-localisation, in a similar manner to their co-localisation in focal adhesions in cultured cells. Unlike FAK, paxillin immunoreactivity is not found to be consistent throughout the apical epiblast but rather in small groups of cells, the reason for which is unclear.

When FAK and Pyk2 expression *in vivo* were compared, it was found that the latter, in contrast to FAK, was expressed only in very low levels as judged by immunoblotting. Whereas FAK showed a tissue-specific regulation in reaction to

explanting and culturing the cells, Pyk2 showed a more dramatic, but non-tissue specific up-regulation. Clearly, there is differential regulation of these two similar molecules in response to this change in conditions, as there is in response to other stimuli (Matter et al., 1998). Although there was no significant difference in expression between the germ layers, Pyk2 did show similar pattern of expression to FAK in each of the germ layers in the embryo, with the highest expression in the epiblast and the lowest in the endoderm, there appeared to be a lower level of expression of Pyk2 *in vivo*. The level of paxillin expression in each of the three germ layers was increased after explantation of tissues into culture. Paxillin immunoblots showed much larger bands than either FAK or Pyk2 in each layer of the embryo, suggesting a higher level of expression. The transition to *in vitro* conditions, where presumably there are changes in adhesion and the cytoskeleton due to the two-dimensional growth surface, influences the expression of these three proteins differentially showing that they are independently regulated despite their related signaling properties.

On the immunoblots for FAK there are other bands at lower molecular weights that are probably fragments of FAK that have been cleaved by proteolytic enzymes. FAK has been shown to be cleaved by both calpain (Cooray et al., 1996), and members of the caspase family (Wen et al., 1997) and this breakdown is thought to be a possible means of regulation of FAK activity. The molecular weight of the bands present on the immunoblots is similar to that seen in studies examining FAK cleavage fragments and is not likely to be the autonomously expressed protein FRNK, that is identical to the C-terminal domain of FAK. The antibodies to FAK used in these studies are all raised against C-terminal sequences of FAK and should therefore be able to detect not only the full length FAK protein but also FRNK. In the work presented here there was no detectable expression of FRNK in chick embryo cells, either *in vivo* or *in vitro*. The expression of FRNK is thought to be a mechanism for negative regulation of FAK signaling by competing for common binding proteins (Richardson and Parsons 1996). The fact that cells from gastrulation stage chick embryos do not show any significant expression of FRNK suggests this is not a means of regulation used in these cells and perhaps the presence of potential FAK breakdown products reflects the degradation of FAK as an alternative means of regulation. It is interesting to note that there is a significant band present in the Pyk2

immunoblots at around 43 kDa that appears in tissues from both the *in vivo* and *in vitro* conditions. As for FAK, Pyk2 appears to have an autonomously expressed C-terminal fragment (PRNK; Xiong et al., 1998), that is likely to be the 43 kDa band present in these immunoblots. If PRNK is involved in the regulation of Pyk2 signaling, in a similar manner to which FRNK regulates FAK, the presence of PRNK and lack of FRNK expression indicates a possible difference in the regulation of FAK and Pyk2 in chick embryo cells.

Attempts to determine the signaling molecules that interact with FAK and are therefore likely involved in FAK signaling have lead to a number of proteins being identified as FAK binding proteins in cultured cells. Paxillin is a direct substrate for FAK and the phosphorylation of paxillin by FAK is thought to initiate the formation of a signaling complex arranged around paxillin (Schaller and Parsons 1995). To try and determine if FAK interacts with similar signaling molecules *in vivo* as it does in culture, work here was done to co-precipitate FAK with its associated proteins under native conditions from both *in vivo* and *in vitro* cells. If, *in vivo*, FAK associates with the same proteins that are found to co-localise with FAK in focal adhesions it would suggest that although focal adhesions have not been observed *in vivo* the same signaling pathways may be present, only not arranged in adhesive sites. After immunoprecipitation of FAK under native conditions no association with paxillin or Src could be observed in samples from either *in vivo* or in *vitro* conditions. This result is somewhat inconclusive as it would be expected that FAK would be associated with paxillin from *in vitro* samples as this has been observed in other cell types (Hildebrand et al., 1995). Since all the results under the different conditions used were negative results showing no interaction of these proteins either FAK is not associated with paxillin or Src *in vivo* or *in vitro* in chick embryo cells or the lack of paxillin or Src association with FAK may reflect a weak association that was disrupted even under the native conditions used in the experiment.

There are several areas of cell behavior that could be influenced by integrin activated signaling through FAK. Signaling to the MAPK cascade (Schlaepfer et al., 1994) could regulate cell growth, signaling to the cytoskeleton and adhesive sites could regulate cell spreading and migration (Fincham and Frame 1998) and signaling through other pathways could regulate apoptosis (Frisch et al., 1996). After treatment with sense or

antisense oligonucleotides the levels of apoptosis and cell spreading were examined to determine if FAK was involved in either of these phenomenon.

There are several problems associated with the use of antisense oligonucleotides. It is necessary to show that the oligonucleotides have successfully been introduced into a sufficient number of cells to observe an effect and also to show that any observed effect is specifically due to inhibition of mRNA function by the antisense oligonucleotide. The first problem has been overcome in these experiments by the use of biotinylated oligonucleotides. This allows the treated cultures to be fixed and oligonucleotides visualised using FITC-conjugated streptavidin. The results showed that sense and antisense oligonucleotide-treated cultures showed a high percentage of the cells labeling positively for oligonucleotide compared to the untreated or cytofectin-treated cultures, which showed no labeling. The high number of cells labeled for oligonucleotide suggests that there would be a large enough effect of the oligonucleotide to have an observable effect and also, any effect is likely to be uniform throughout the culture.

The immunoblots showed that the levels of FAK protein expression were reduced by 70% in antisense oligonucleotide-treated cultures compared to the expression of FAK in sense oligonucleotide-treated or untreated cultures. This suggests there is a specific effect of the antisense oligonucleotide on FAK protein expression. Also, the fact that the levels of Pyk2 and paxillin protein were not affected shows that the inhibition of FAK expression was specific and that there was not an overall reduction in protein synthesis through effects on cell metabolism or non-specific induction of cell death.

The reduction in levels of FAK expression does not appear to have a direct influence on the levels of apoptosis in the cell cultures examined here. Other studies have shown an increase in the levels of apoptosis in tumor cells after attenuation of FAK expression with antisense oligonucleotide-treatment (Xu et al., 1996). However, in the same study, there was no effect of antisense oligonucleotide-treatment on the levels of apoptosis in normal cells. This correlates with the results here where there is no clear effect of the antisense oligonucleotide to FAK on apoptosis. The studies here show that when chick embryo cells are grown in serum-free conditions there is no change in the expression of FAK or Pyk2, even though there is a significant rise in the number of

apoptotic cells under these conditions. This suggests the increase in apoptosis is not due to a reduction in the expression of either of these proteins. The cleavage of FAK by caspases has been reported to be an early event in apoptotic cells (Crouch et al., 1996) but it appears that there is no measurable cleavage of FAK or Pyk2 in the serum-starved cultures.

The fact that the majority of the cells in the culture survive the growth period in serum-free medium suggests there are other factors that are providing the cells with survival signals that prevent the cells from undergoing apoptosis. One source for this survival signal may be the ECM. Adhesion to fibronectin has been shown to prevent apoptosis in some cell types (Aoshiba et al., 1997b; Scott et al., 1997) although it is still not clear which signaling pathway is regulating this process and there may be more than one pathway involved. If the ECM, which in these experiments was fibronectin, is providing a survival signal for the serum-starved cells, is FAK involved? Cells grown in serum-free medium and also treated with antisense oligonucleotides to FAK show no significant increase in apoptosis compared to untreated and sense oligonucleotide-treated cultures grown in serum-free conditions. This suggests there is an alternative pathway that does not involve signaling through FAK that can prevent apoptosis in these cells. FAK has been shown to be involved in regulating cell survival in some cells (Hungerford et al., 1996), although there are other pathways activated by integrins that regulate anti-apoptotic proteins such as Bcl-2 that may regulate apoptosis in some cells (Zhang et al., 1995b). Signaling through alternative pathways such as this may regulate apoptosis in these cells, as there is no apparent involvement of FAK.

Treatment of cells with antisense oligonucleotide to FAK did result in a change in cell shape that was reflected by a change in the distribution of focal adhesion sites and the actin cytoskeleton. The morphology of the cells after antisense oligonucleotide-treatment suggested an inability of the cells to spread and possibly migrate. Cells from FAK knockout mouse embryos were found to have a defect in migration when examined in culture (Ilic et al., 1995a). This is similar to the results seen here in chick embryo cells where the cells show a reduced level of polarisation and smaller area, suggesting the cells may lack the ability to regulate adhesion or the cytoskeleton in a manner that promotes

spreading and migration. The actin cytoskeleton present in untreated or sense oligonucleotide-treated cells showed a general polarisation of the stress fibres so that they were aligned in the same direction. This shows a generation of tension within the cell that aligns the stress fires and produces a polarised morphology in the cell (Burridge and Chrzanowska-Wodnicka 1996). The bundles of actin around the periphery of antisense oligonucleotide-treated cells is characteristic of non-motile cells and this type of actin is known to be associated with peripheral focal adhesions (Small et al., 1998). The antisense oligonucleotide-treated cells showed a distribution of focal adhesions mainly around the periphery of the cells, which are likely to be associated with the actin bundles seen in these cells. The fact that there are changes in the architecture of the actin cytoskeleton suggest there may be a disruption of downstream signaling to the actin cytoskeleton in the cells treated with antisense oligonucleotides to FAK. The small G-protein Rho is an important regulator of the cytoskeleton and Rho activation is known to stimulate the activation of FAK (Flinn and Ridley 1996). Conversely, FAK has also been shown to influence Rho activation through its association with the GTPase regulator associated with FAK (GRAF), leading to changes in the actin cytoskeleton (Taylor et al., 1999). The reduced levels of FAK in antisense oligonucleotide-treated cells may lead to changes in an FAK/Rho signaling pathway that results in the observed loss of stress fibres and rounding of the cells. A secondary effect of the changes in the cytoskeleton after antisense oligonucleotide-treatment may be a change in the cells' ability to respond to mechanical stimuli that would normally be detected through a tensegrity model (Ingber 1997, see Chapter 2).

In conclusion, these results show a spatio-temporal relationship between FAK expression and the events of gastrulation, and have demonstrated that the various substrata encountered by the differentiating cells during gastrulation are able to influence the expression and levels of tyrosine phosphorylation of this molecule. The results support the fact that FAK appears to play a significant role in the gastrulation process (Furuta et al., 1995; Ilic et al., 1995a). FAK does not appear to localise to sites of adhesion on the cell surface *in vivo*, although it localises to focal adhesion sites *in vitro*. Despite the structural similarities between FAK and Pyk2, and the association of both FAK and

paxillin with focal adhesions, each of these three molecules are independently regulated and respond differentially when subjected to *in vitro* conditions. Taken together these results suggest the organisation of FAK and other signaling molecules associated with signaling from sites of adhesion may not be organised into localised sites of adhesion *in vivo*. The change to *in vitro* culture conditions changes the expression level of each protein examined here and may also affect their localisation through changes in the cytoskeleton and adhesion. Although there are differences in expression and localisation of these proteins *in vivo* and *in vitro*, the same signaling pathways may be activated in response to adhesion through integrins. Functional studies suggest FAK is not involved in regulation of apoptosis in chick embryo cells but may be involved in regulating the cytoskeleton and cell spreading, important in cell migration within the embryo.

FUTURE DIRECTIONS

The majority of the experiments conducted here were done using cells taken from gastrulation stage chick embryos that were grown *in vitro*. The assumption was made that the cells would behave in a similar manner to those *in vivo* in terms of adhesive behavior and signaling activity. However, results show that the organisation of adhesive sites and the levels of protein expression appear different *in vivo* and *in vitro*. Future studies addressing the function of FAK and associated signaling molecules on *in vivo* embryonic models would be useful in understanding the role of these proteins in early developmental stages. The use of the antisense oligonucleotide treatment developed here to deliver oligonucleotides to gastrulating embryos or treatment of dissected tissues that could be explanted back into the embryo could be used to examine the effects of reduced FAK expression on cell survival and morphology as the cells migrate in the embryo.

There is also the possibility of using recombinant adenoviral vectors to overexpress FAK protein. The availability of the vectors containing the full-length FAK gene allows for over expression of FAK in both *in vitro* and *in vivo* conditions. This provides another means of examining the function of FAK in the embryo where interaction of cell types and the three dimensional forces of the embryo may influence adhesion signaling.

The fact that mouse embryos lacking FAK expression do not advance beyond late gastrulation stages (Furuta et al., 1995) prevents the examination of FAK function in later

development in these embryos. Treatment of specific regions of the embryo or transplanted tissue with antisense oligonucleotide to FAK or adenoviral vectors with the FAK gene might allow analysis of the function of FAK at later stages of development.

The nuclear localisation of FAK described in these studies is unusual although there are a growing number of adhesion-related proteins with apparent nuclear localisations. Further studies to confirm the localisation of FAK in the nucleus by fractionation of the nuclei from cell lysates would be beneficial.

It would also be of interest to examine the involvement of Rho in FAK signaling during development. The results presented here suggest that FAK signaling has effects on the cytoskeleton and Rho may be an effector of FAK signaling to the cytoskeleton since FAK can influence Rho activity through the GTPase Graf.

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